IDIOSYNCRATIC APPORCH FOR AN ERRATIC AND STUNNING MANAGEMENT OF *Aegle marmelos* Linn. LEAVES ON PHOTOSENSITIVITY BY USING ZEBRA FISH EMBRYO MODEL

Dissertation submitted to

The Tamil Nadu Dr. M.G.R. Medical University

Chennai-600 032

In partial fulfillment of the requirements

For the award of the degree of

MASTER OF PHARMACY
IN
BRANCH – III – PHARMACOGNOSY

Submitted by
S.RAJASEKAR
Reg. No.261620706

Under the guidance of

Dr. A. SETHURAMANI, M.Pharm., Ph.D.,

Department of Pharmacognosy

COLLEGE OF PHARMACY

MADURAI MEDICAL COLLEGE

MADURAI - 625 020

MAY-2018

CERTIFICATE

This is to certify that the dissertation entitled **"IDIOSYNCRATIC APPORCH FOR AN ERRATIC AND STUNNING MANAGEMENT OF *Aegle marmelos* linn LEAVES ON PHOTOSENSITIVITY BY USING ZEBRAFISH EMBRYO MODEL*)*"** is a bonafide work done by Mr. S.RAJASEKAR (261620706) , **DEPARTMENT OF PHARMACOGNOSY , COLLEGE OF PHARMACY , MADURAI MEDICAL COLLEGE, MADURAI -625020** in partial fulfillment of the Tamilnadu Dr. M.G.R Medical University rules and regulation for award of **MASTER OF PHARMACY IN PHARMACOGNOSY** under my guidance and supervision during the academic year 2017- 2018.

Name& signature of the Guide: (A.SETHURAMANI)

Name & signature of the Head of the Department:

Name & Signature of the Dean/ Principal:

Dr. D.STEPHEN, M.Sc., Ph.D.,
ASSISTANT PROFESSOR

DEPARTMENT OF BOTANY
THE AMERICAN COLLEGE
MADURAI-625002

CERTIFICATE

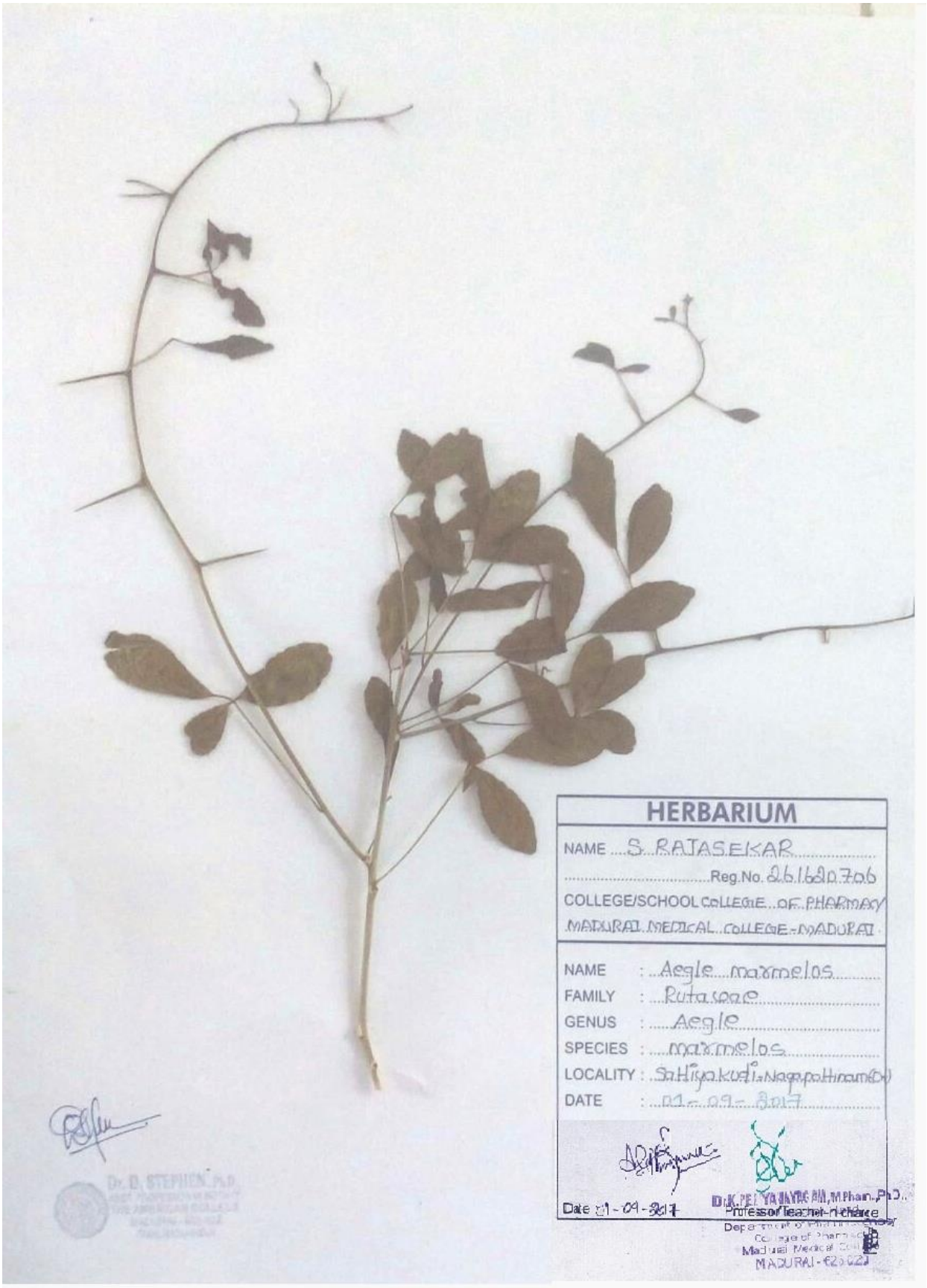
This is to certify that the specimen brought by Mr.S.Rajasekar, II M.Pharm, Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai is identified as *Aegle marmelos Linn.* belonging to the family Rutaceae.

Station : Madurai.

(Dr.D.STEPHEN)

Date : 17.08.2017.

Dr. D. STEPHEN, Ph.D.,
ASST PROFESSOR IN BOTANY
THE AMERICAN COLLEGE
MADURAI - 625 002
TAMILNADU INDIA



HERBARIUM

NAME ....S. RAJASEKAR.....

.....Reg.No. 26.1.6.d.0.7.0.6

COLLEGE/SCHOOL COLLEGE OF PHARMACY

MADURAI MEDICAL COLLEGE - MADURAI

NAME : Aegle marmelos

FAMILY : Rutaceae

GENUS : Aegle

SPECIES : marmelos

LOCALITY : Sathiyakudi - Nagappa Hill (Dt)

DATE : 01-09-2017

Date 01-09-2017

Dr.K. PERIYANAYAGAM, M.Pharm., Ph.D.,
Professor & Teacher-in-charge
Department of Pharmacognosy
College of Pharmacy
Madurai Medical College
MADURAI - 625 020

Dr. D. STEPHEN, Ph.D.

Manonmaniam Sundaranar University
Constituent Model College,
NAGALAPURAM TK,
Thoothukudi TK. 628904

Sub:

Your Ref:

Our ref:

Date:

Prof. (Major) P.Chanra Sekaran,
Principal. MSU Constituent Model College
Nagalapuram 628 904.
Formerly Faculty of PG & Research
Department of Zoology and Biotechnology
Vivekananda College (Autonomous)
Tiruvedakam west 625217.

CERTIFICATE

This is to certify that the specimen brought by Mr.S.Rajasekar II M.Pharm. Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai has been identified as, "*Danio rerio*", family: Cyprinidae

Station: Madurai

Date:

Prof (Major) P.CHANDRASEKARAN.
PRINCIPAL,
MS University Constituent Model College.
NAGALAPURAM,
Vilathikulam Taluk Thoothukudi Dt 628 904

E-Mail id:
Phone: 04638-242666 majorpcs@yahoo.co.in Fax No:

ACKNOWLEDGEMENT

I first and foremost express my revered regard and obeisance to the **ALIMIGHTY GOD** with whose blessings I was able to complete my project work.

I am grateful to express my sincere thanks to **Dr. D. MARUTHUPANDIAN, M.S., FICS., FAIS.,** Dean, Madurai Medical College, Madurai, for giving an opportunity to carry out my project work.

I am thankful to **Dr. V. DHANALAKSHMI, M.D.,** Vice Principal, Madurai Medical College, Madurai for her support and encouragement to carry out the work.

I express my thanks to Prof. **Dr. A. ABDUL HASAN SATHALI**, **M.Pharm., Ph.D.,** Principal and Head of Department of Pharmaceutics, College of Pharmacy, Madurai Medical College, Madurai for his support and valuable suggestions.

I express my sincere thanks to **Dr. K. PERIYANAYAGAM, M.Pharm., Ph.D., P.G. Diploma in Clinical Pharmacy (Australia),** Professor and Head of Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai for his support and valuable suggestions.

It is my privilege to express a deep and heartfelt sense of gratitude and my regards to our respected **Dr.A.Sethuramani, M.Pharm., Ph.D.** Assistant professor, Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai for her active guidance, advice, help support and encouragement. I am very much obliged for her perseverance, without which my project work would not be completed.

I thank **Mrs**.**A.KRISHANAVENI M.Pharm., Mr.G. SATHYA BALAN M.Pharm., Mr.M.R.VINAYAKAMURTHI, M.Pharm.** Assistant professor in Pharmacognosy, Department of Pharmacognosy, College of Pharmacy, Madurai Medical College, Madurai for their help.

I am thankful to **Mrs.K.RADHA**, Lab supervisor**, Mrs.A.CHITRA,** Lab technician for their support during my study of this work.

I thank to **Dr. D. STEPHEN, M.Sc., Ph.D.,** Department of Botany, American College, Madurai for Herbarium identification.

I thank to **profs, P.CHANDRASEKARAN**, Department of Zoology and Biotechnology, Vivekananda college, Thooothkudi ,for identification of animal specimen.

I special thanks to my classmates **Mrs.L.SRIKALA, Mrs.R.SUGANYA, Mrs.G.HEMALATHA, Miss.D.SANGEETHA, Mr.T.PRABHAKARAN, Mr.B.EZHILARASAN, Mr.M.MOHANRAJ, Mr.A.IYAPPAN** for helping my project.

I thank my M.pharm pharmacognosy juniors **Miss.P.BRINDHA, Miss.A.DEEBIKA, Miss.R.DHANALAKSHMI, Mr.B.KARTHIKEYAN, Mrs.P.KOHILAVANI, Mr.K.MUTHUKRISHNAN, Mr.V.RAJAMANICKAM, Mrs.J.SELVARANI, Mr.VIJAYAGANESH** for helping my project.

I extend my thanks to all the teaching and non-teaching staffs of other departments of College of Pharmacy, Madurai Medical College, Madurai who have rendered their help for the completion of the project.

CHAPTER NO	TITLE	PAGE NO
	LIST OF TABLES	
	LIST OF PLATES	
	LIST OF FIGURES	
1	**INTRODUCTION**	1
2	**LITERATURE OF REVIEW**	16
	Morphology and occurrence of *Aegle marmelos* Linn	19
	Traditional uses of *Aegle marmelos* Linn	19
	Pharmacognostical Review of *Aegle marmelos* Linn	20
	Pharmacological Review of *Aegle marmelos* Linn	22
	Phytochemical Review of *Aegle marmelos* Linn	25
3.	**AIM AND OBJECTIVE**	26
4	**MATERIALS AND METHODS**	26
	Pharmacognostical studies	29
	Collection and identification of plant material	31
	Macromophological evelauation of *Aegle marmelos* Linn	34
	Transaction of *Aegle marmelos* Linn	35
	Sectioning	36
	Photomicrographs	41

3	Quantitative Measurements of *Aegle marmelos* Linn	43
	Preparation of powder of *Aegle marmelos* Linn leaves	43
	Physicochemical Evaluation of *Aegle marmelos* Linn leaves	43
	Fluorescent Analysis	46
	Qualitative Phytochemical Analysis of *Aegle marmelos* Linn leaves	48
	Quantative phytochemical Estimation of *Aegle marmelos* Linn leaves	50
	Chromatography	47
	Column chromatography of *Aegle marmelos* Linn leaf extract	48
	Isolation Identification and characterization	60
	UV Spectroscopy	62
	IR Spectroscopy	62
	PHARMACOLOGICAL STUDIES	
	Reducing power assay	65
	Total antioxidant activity by Phosphomolybdenum Method.	66
	In vitro sun protection factor determined by UV -Vis spectroscopy method.	69
	Toxicological studies of EEAM on the early development of zebra fish Embryo toxicity study	72
	In vivo photo protective activity against UVA radiation using zebra fish embryo model.	73

5.	**RESULTS AND DISCUSSION**	75
	Pharmacognostical studies of *Aegle marmelos* Linn leaves	
	Morphological studies leaves of *Aegle marmelos* Linn leaves	75
	Microscopical studies of *Aegle marmelos* Linn leaves	77
	Powder microscopical Characteristics of *Aegle marmelos* Linn leaves	79
	Quantitative Measurements *Aegle marmelos* Linn leaves	80
	Physicochemical Evaluation of *Aegle marmelos* Linn leaves	81
	Fluorescent analysis	83
	Phytochemical studies	
	Preliminary Phytochemical screening	85
	Quantative phytochemical Estimation	87
	Column chromatography	95
	Isolation, Identification and purification of Bioactive compounds	97
	UV Spectroscopy	99
	IR Spectroscopy	100
	Pharmacological studies	
	In vitro anti-oxidant activity	
	Reducing power assay	103
	Total antioxidant activity by Phosphomolybdenum Method.	105

	In vitro sun protection factor determined by UV -Vis spectroscopy method.	107
	Toxicological studies of EEAM on the early development of zebra fish Embryo toxicity study	110
	In vivo photo protective activity against UVA radiation using zebra fish embryo model.	110
6	**SUMMARY AND CONCLUSION**	115
7	**REFERENCE**	119

S.NO	PARTICULAR	PAGE NO
1.	Macroscopial studies of *Aegle marmelos* linn leaves	76
2	Quantitative microscopy of *Aegle marmelos* linn leaves	80
3.	Determination of physio- chemical constants of *Aegle marmelos* linn leaves:	81
4.	Organoleptic character of *Aegle marmelos* linn leaves powder	82
5.	Behavioral characters of *Aegle marmelos* linn leaves (Crude powder) with different reagent	83
6.	Fluorescence analysis of extracts of *Aegle marmelos* linn extract	84
7.	Preliminary phytochemical screening of *Aegle marmelos* linn leaf Powder and different extracts	85
8.	Total phenolic content in ethanolic extract *Aegle marmelos* linn leaves of in terms of Gallic acid equivalents	88
9.	Total flavonoid content per gram of extract in terms of Rutin By aluminum chloride method	90
10.	Total tannin content in ethanolic extract of *Aegle marmelos* linn in terms of Tannic acid equivalents	92
11.	Total coumarin content in ethanolic extract of *Aegle marmelos linn* In terms of coumarin equivalents	93
12.	TLC Profile of *Aegle marmelos* linn leaves	95
13.	Data showing the column chromatography of EEAM	96
14.	Nature and properties of isolated compound from chloroform – ethanol fraction of EEAM	97
15.	Thin layer chromatography profile of isolated compound	97

16.	Chemical test for isolated fraction chloroform: ethanol fraction	98
17.	Absorption spectrum value of isolated compound from EEAM.	99
18.	The IR spectrum observed ranges of isolated compound from EEAM	101
19.	Absorbance of ethanolic extract of *Aegle marmelos* linn leaves and standard ascorbic acid in reducing power assay:	104
20.	Absorbance of ethanolic extract of *Aegle marmelos* linn leaves and standard ascorbic acid in phosphomolybdenum assay	105
21.	SPF of ethanolic extract of *Aegle marmelos linn* leaves: For 2 mcg /ml concentration	107
22.	SPF of ethanolic extract of *Aegle marmelos linn* leaves: For 4 mcg /ml concentration	108
23.	SPF of ethanolic extract of *Aegle marmelos linn* leaves: For 6 mcg /ml concentration	108
24.	SPF value standard : For 25 mcg /ml concentration	109
25.	Malformation Score For the Whole Embryo Toxicity For EEAM	110
26.	Malformation Score For the Whole Embryo For Dmso	111
27.	Malformation Score For the Whole Embryo For Standard Caffeine	111
28.	Zebrafish embryo Malformation changes for Control	112
29.	Zebrafish embryo Malformation changes for positive control	112
30.	Zebrafish embryo Malformation changes for extract	113
31.	Malformation changes for standard	113

FIGURE CONTENT

S.NO	PARTICULAR	PAGE.NO
1.	Calibration curve of Gallic acid	87
2.	Calibration curve of Rutin	89
3.	Calibration curve of tannic acid	91
4.	Calibration curve of coumarin	94
5.	Absorption spectrum of isolated compound	99
6.	IR Spectrum of isolated compound	100
7.	Graphical representation of absorption of reducing power assay of ascorbic acid	103
8.	Graphical representation of absorption of reducing power assay of EEAM	103
9.	Graphical representation of absorption of total antioxidants assay of ascorbic acid &EEAM	105

PLATE CONTENT

S.NO	PARTICULAR
1	Habitat
2.	Dorsal View Of Aegle Marmelos Linn Leaves
3	Ventral View Of Aegle Marmelos Linn Leaves
4	T.S Of Midrib Of Leaves Aegle Marmelos Linn
5	T.S Of Lamina Of Leaves Aegle Marmelos Linn
6	T.S Of Petiole Of Leaves Aegle Marmelos Linn
7.	Stomata
8.	Venation Pattern Of Aegle Marmelos Linn Leaves
9.	powder microscopy of aegle marmelos linn leaves
10.	Tlc Profile Of EEAM –Mobile Phase -1
11.	Tlc Profile Of EEAM –Mobile Phase -2
12.	Tlc Images Of Isolated Compound
13.	Zebrafish Embryo Malformtion Images For Standard
14	Zebrafish Embryo Malformtion Images For EEAM
15.	Zebrafish Embryo Malformtion Images For Dmso
16.	Zebrafish Embryo Malformation Images For Egg Water
17.	Zebrafish Embryo Normal Development

\

CHAPTER-1

INTRODUCTION

CHAPTER-1

INTRODUCTION:

Pharmacognosy: is the study of medicinal properties of traditionally used herbs in their crude or unprepared form. The word derived from the Greek word pharmakon (drug), and gnosis or (knowledge).

Pharmacognosy is a subject to identify or authenticate the plant parts using Macroscopial , anatomical & phytochemical characters ,and it includes the study of the physical , chemical , biochemical and biological properties of drugs, drug substances or potential drugs or drug substances of natural origin as well as the search for new drugs from natural sources .Although pharmacognosy is principally concerned with plant materials , there are some animal products which are traditionally encompassed within the subject such as (beeswax , gelatin , wool fat ,Vitamins etc.. In addition to that the antibiotics , hormones and others may be also involved , Marine organisms (Plants & Animals) which have special potent pharmacological actions are now paied high attention in the search for new drugs .There are some materials having no pharmacological action but have interest to pharmacognosists are (Natural fibers , Flavoring and suspending agents , colorants , disintegrants , stabilizers , and filtering and supporting media . Also there are other materials that have natural associations with the subject are (Poisonous & Hallucinogenic plants , allergens herbicides , insecticides) .

Traditional medicine has remained as the most affordable and easily accessible source of treatment in the primary health care system of resource poor communities. The local people have a long history of traditional plant usage for medicinal purposes. The medicinal use of

plants is very hoary. The writings indicate that therapeutic use of plants is as old as 4000 - 5000 B.C. and Chinese used first the natural herbal preparations as medicines. In India, however, earliest references of use of plants as medicine appear in Rig-Veda, which is said to be written between 1600 - 3500 B.C. Later the properties and therapeutic uses of medicinal plants were studied detail and recorded empirically by the ancient physicians (an indigenous system of medicine) which are a basic foundation of ancient medical science in India Medicinal plant is an important element of indigenous medical systems in all over the world. The ethno botany provides a rich resource for natural drug research and development.

"Traditional" use of herbal medicines implies substantial historical use, and this is certainly true for many products that are available as "traditional herbal medicines". In many developing countries, a large proportion of the population relies on traditional practitioners and their armamentarium of medicinal plants in order to meet health care needs. Although modern medicine may exist side-by-side with such traditional practice herbal medicines have often maintained their popularity for historical and cultural reasons. Natural products have played an important role throughout the world in treating and preventing human diseases. Natural produce medicines have come from various source materials including terrestrial plants, terrestrial microorganism, marine organisms, and terrestrial vertebrates and invertebrates and its importance in modern medicine has been discussed in different reviews and reports. The value of natural products in this regard can be accessed from:1) the rate of introduction of new chemical entities of wide structural diversity, including serving as templates for semi synthetic and total synthetic modification, 2) the number of diseases treated or prevented by these substances, and 3) their frequency of use in the treatment of disease. In recent years, the use of traditional medicine information on plant research has

again received considerable interest. In recent times, there have been increased waves of interest in the field of research in natural products chemistry. This level of interest can be attributed to several factors, including unmet therapeutic needs, the remarkable diversity of both chemical structure and biological activities of naturally occurring secondary metabolites, the utility of novel bioactive natural compounds as biochemical probes, the development of novel and sensitive techniques to detect biologically active natural products, improved techniques to isolate, purify, and structurally characterize these active constituents, and advances in solving the demand for supply of complex natural products.

MEDICINAL PLANT:

Plants containing inborn potentially active ingredients used to cure disease or relieve pain are called medicinal plants. Plants play a therapeutic and restorative role in protecting human beings from the adverse effects of diseases and other complications, thus considered to have a beneficial role in healthcare system. That is the reason that large proportion of population of the developing countries still rely on herbal medicines. Despite their importance, medicinal plants are seldom handled within an organized manner and most are of them are exploited with little or no respect for the future (Srivastava *et al*., 1996; Nair *et al*., 2005). Significant increase in medicinal plants usage has been recorded continuously both for traditional users and pharmaceutical industry. Medicinal plants provide opportunities for biological screening, methods useful for the industry and trends in the pharmacological investigations of natural products. Plants are the natural and most easy accessible source of therapeutically active biological principles, thus there is a dire need to screen out plant for development of new drugs. For this purpose plants have been assayed widely but still large number of them has not arrived to the conventional health care system (Esimone *et al*, 2003;

Therefore, search for new drugs from microorganisms, fungi, plants and animals must be persistent and these can be the sources of innovative and prevailing restorative agents for newer, safer and accessible drugs. Now a day, due to advancement of modern and new sophisticated methods, plant scientists are taking more in trust in exploring new drugs from natural and biologically active compounds of the plants, which could be serve as inexhaustible resources for pharmaceutical industries. Pakistan has a unique position among developing countries, having about 6000 taxa of angiospermic plants including a variety of medicinal plants due to variation in topographic conditions. Moreover it is interesting to say that about 50% of the population in Pakistan is being treated with local herbal preparations by almost 50,000 hakims (traditional herbal practitioners). More than 350 herbal items (as whole herbs or with specific parts) have been reported, which are used in Unani herbal preparations by various Dawakhanas (herbal drugs manufacturing laboratories) in Pakistan. Pakistani flora offers great opportunity for the discovery of new bioactive compounds for various ailments.

The contribution of medicinal plants to modern medicine:

The factor which emphasizes this attention is the incidences of harmful nature of synthetic drugs which are regarded as harmful to human beings and environment. Medicinal plants have been an integral part of the Chinese, Indian and Arabian ancient culture as medicine and their importance even dates back to the Neanderthal period. In spite of this, we discerned about history and traditional uses of medicinal plant by common people. In the 18th century knowledge about plant derived drugs expanded, but attempts to identify the active ingredients from plants were unsuccessful. Another achievement in the field of medicinal plants was the development of methods to study the pharmacological effect of natural products and vegetable extracts. Claude Bernard (1813-1878), who conducted detailed

studies on the pharmacological effects of curare (a drug and arrow poison used by the American Indians of the Amazon), is considered one of the first scientists in this field. The 20th century saw the integration of ethnobotanical, pharmacological and phytochemical studies, a process that had taken many and many years, but which allowed the development of a new approach to the study the significance of medicinal plants in pharmaceutical field.

Protagonist of World Health Organization (WHO) in Phytomedicine:

The multitude of people on this planet still relies on their traditional *Materia Medica* (medicinal plants and other materials) for their everyday health care needs. It is also a fact that one quarter of all medical prescriptions are formulations based on substances derived from plants or plant-derived synthetic analogs, and according to the World Health Organization (WHO), 80 % of the world's population primarily those of developing countries rely on plant-derived medicines for their primary healthcare needs. WHO states that this "traditional medicine" implies the knowledge and practices of herbal healing for the prevention, diagnosis, and elimination of physical, mental, or social imbalance. About 25% of the drugs prescribed worldwide come from plants, 121 such active compounds being in current use. Of the 252 drugs considered as basic and essential by WHO, 11% are exclusively of plant origin and a significant number are synthetic drugs obtained from natural precursors.

Current status of herbal remedies in health care system:

According to WHO, more than 80 % of the world's population, mostly in poor and less developed countries depends on traditional plant-based medicines for their primary healthcare needs? Lag phase for plant based medicine is now rapidly changing for a number of reasons. Problem with drug resistant microorganism, side effects of modern drugs and emerging diseases for whose no medicines are available, have stimulated renewed interest in

plants as a significant source of new medicines34. However, the last few years have seen a major increase in their use in the developed world because of better cultural acceptability, better compatibility with the human body and lesser side effects35. Currently, approximately 25% of drugs are derived from plants, and many others are synthetic analogues built on prototype compounds isolated from plant species in modern pharmacopoeia36. Plants are important sources of medicines and presently about 25% of pharmaceutical prescriptions in the United States contain at least one plant-derived ingredient. In the last century, roughly 121 pharmaceutical products were formulated based on the traditional knowledge obtained from various sources. Today, natural products and their derivatives represent about 50 % of all drugs in clinical use, with higher plant-derived natural products representing commonly approximately 25 % of the total.

Global burden Diseases:

In day to life human has suffered from communicable & non communicable diseases. They are cancer, Tb, dengue, diabetes, CVS related diseases and many infectious diseases. From the above cancer type of melanoma, non-melanoma skin cancer, skin cancer are emerging one now days.

Skin diseases:

The skin care has exposed tremendous growth and emerging one in recent years. In recent studies 10 most emerged and burden skin diseases globally affect the life. These are dermatitis, acne, hives, psoriasis, viral skin disease, fungal skin diseases, scabies, melanoma, pyoderma, cellulitis, non-melanoma skin cancer, decubitus, and alopecia areata. (IJD Syposium 2017)

. The skin diseases in India 10-12% of total population affected with eczema, psoriasis being major problem. Due to pollutions, ultraviolet radiations, and global warming, photosensitive skin disorder like danning, pigment darkening, sunburn, skin cancer.one percent of damage in ozone leads to four percent of skin tumors' rate.

The world health organization classify the current diseases system

1. Heart, lung and other organ disease
2. Blood and immune system disease
3. Cancer
4. Injury
5. Brain and nervous system diseases
6. Endocrine system diseases
7. Infection and parasitic diseases
8. Pregnancy and child birth related diseases
9. Inherited diseases
10. Environmentally- Acquired diseases

The last one environmentally acquired diseases caused by the environmental factor such as heat, radiation waves, and gases. The diseases are skin cancer, carbon- monoxide poisoning, etc. The skin diseases in India 10-12% of total population affected with eczema, psoriasis being major problem. Due to pollutions, ultraviolet radiations, and global warming, photosensitive skin disorder like danning, pigment darkening, sunburn, and skin cancer.one percent of damage in ozone leads to four percent of skin tumors' rate. (Bio spectrum bureau 2014).

Exposure to ultra violet radiation is a major risk factor most skin cancers. Sun light is the main source of UV rays. Tanning lambs and beds are another source of UV rays. People who get a lot of UV exposure from these sources are at greater risk for skin cancers. Even though UV rays make upon only a very small portion of the sun rays, but they are the main damaging effect of skin and damage the DNA of the skin cells.

Ultraviolet radiation is defined as that portion of electromagnetic spectrum between x rays and visible light .The uv spectrum is divided into vacuum uv (40-190nm), far uv (190-220nm), ultraviolet C (220-290), UV B (290-320), UV A(320-400nm).UV radiation reach the earth surface 95-98%, uv A radiation 2-5% and UV B, UV C absorbed in ozone layer.

UV rays:

UVA RAYS: Age the skin cells and can damage the DNA. These rays linked to the long term skin damage such as wrinkles, but they also thought to play in some skin cancers. Most tanning bed gives of large amount of UVA rays, which has been found to increase skin cancer risk.

UVB RAYS: Have slightly more energy than UVA rays. They can damage the skin cells DNA directly, and are main rays that cause sun burn. They are also cause skin cancer.

UVC RAYS: Have more energy than the other type of UV rays, but they dont get through our atmosphere and are not in sunlight.

The strength of UV rays reaching the earth depends upon the following factor such as:

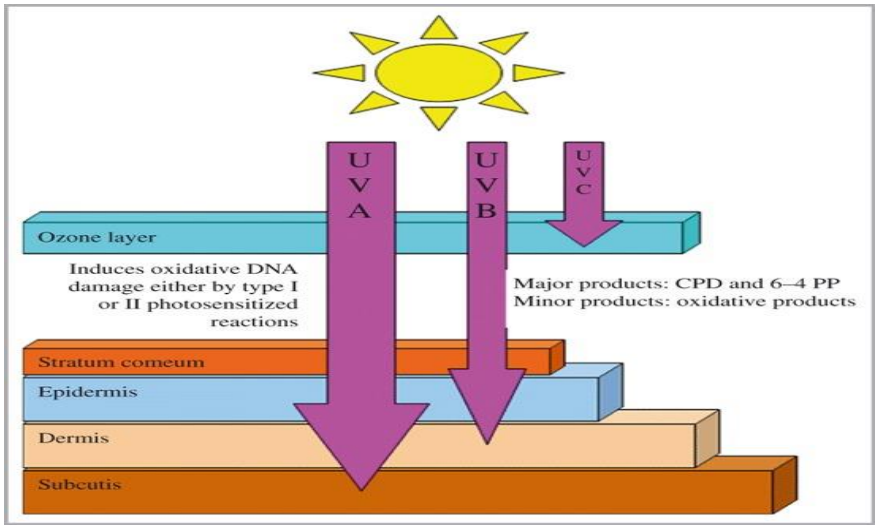
Time of day: UV rays are strongest between 10 am to 4pm.

Season of the year: UV rays are stronger during spring and summer months.

Altitude: More UV rays reach the ground at higher elevations.

Distance from the equator: UV exposure goes down as you get further from the equator

Effects of UV radiation on the skin:

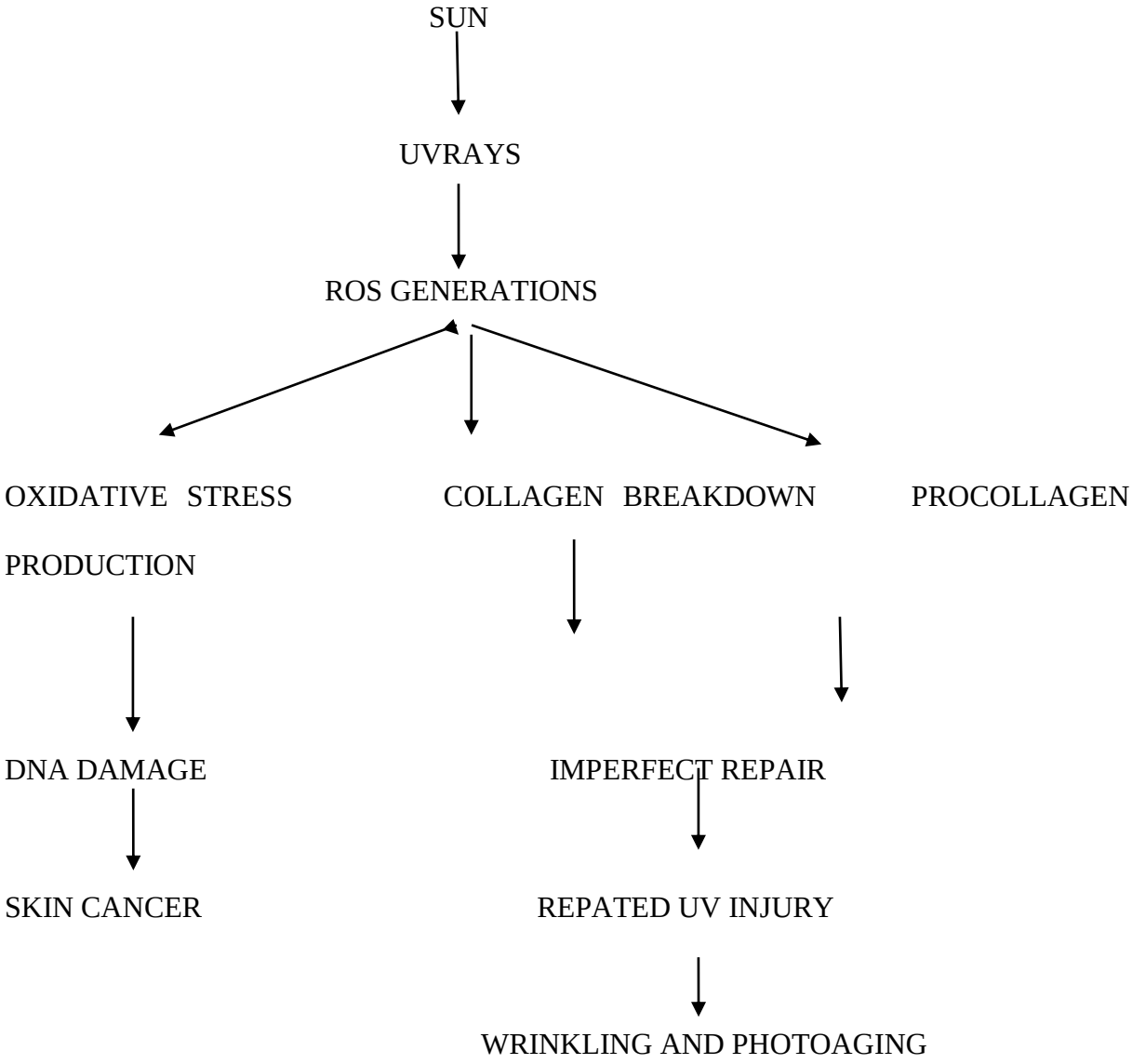


UVC is extremely damaging to the skin. UVC radiation is completely absorbed by molecular oxygen and ozone in the Earth's atmosphere (Afaq *et a*l., 2002).

.UVB radiation, known as burning rays, makes up 4-5%of UV light. UVB can penetrate the epidermis layer of the skin. UVB is 1000 times more capable of causing sunburn than UVA. It induces direct and indirect adverse biological effects, including the formation of pyrimidine photoproducts (Katiyar 2005).

. UVA radiation, known as aging rays, represents more than ninety percent of UV radiation that reaches the Earth's surface (Svobodová *et al.,* 2003). It can penetrate deeper into the epidermis and dermis of the skin (around 1 mm) and produce the generation of reactive structures in the dermis and cause premature photoaging of the skin (Ichihashi *et al.,* 2009). It mainly causes skin sagging rather than wrinkle (Krutmann, 2001).

Photochemical reaction:



Phyto photo protection:

Natural products are the great awareness in drug discovery for the improvement of new therapeutic agent.

Intention for selection of natural photo protective agents:

- No allergic reaction
- Easily formulated
- More effective
- Less cost
- Availability
- Therapeutic good administration (TGA) reported the evidence that zinc oxide and titanium oxide induce free radical formation in presence of light. It causes the photo damage to the normal cells (Mishra AK, 2011).

Current progresses in photo protection:

UV filters (sun screen) is create the physical wall between the sun to the skin. These agents tools for prevent erythema and acute injurious effect of UV exposure and chronic damage, photoaging. Oral photo protectives are shiny missiles in photo protection at the present time.it is the general consensus that they are intended to battle the long term effects of UV exposure, predominantly photoaging and skin tumorigenesis.

CHAPTER-2

LITERATURE REVIEW

CHAPTER-2

LITERATURE REVIEW:

Distribution:

Aegle marmelos is a subtropical plant and grows up to 1, 200 m altitude from sea level. It grows well in the dry forests on hilly and plain areas. It is a widely distributed in India, Ceylon, China, Nepal, Sri Lanka, Myanmar, Pakistan, Bangladesh, Nepal, Vietnam, Laos, Cambodia, Thailand, Indonesia, Malaysia, Tibet, Sri Lanka, Java, Philippines and Fiji. In India it is found in Sub-Himalayan tracts from Jhelum eastwards to West Bengal, in central and south India. It is found almost in all the states of India (Dinesh Kumar Sekar et al 2011). It is also grown in Egyptian gardens in Surinam and Trinidad (Sukhdev, AR 1975).

Habitat:

The tree grows wild in dry forests on hills and plains, also in mixed deciduous and dry dipterocarp forests. It grows up to an altitude of 1, 200 m where the temperature rises to 48.89º C in the shade in summer and descends to -6.67º C in the winter, and prolonged droughts occur. It will not fruit where there is no long, dry season as in southern Malaysia.

PLANT DESCRIPTION;

BOTANICAL CLASSIFICATION:

Kingdom:	Plantae
Sub kingdom:	Trachebiontal
Division:	Magnoliphyta
Class:	Magnoliphyta
Sub class:	Rosidae
Order:	Sapindales
Family:	Rutaceae
Genus*:*	*Aegle*
Species:	*Marmelos*
Synonym:	Beal
Common name:	Bengal quince, Bilva, Golden apple

VERNACULAR NAME:

Bom:	Bael
Ben:	Bela
Eng:	Bael
Guj:	Bilivaphal
Gond:	Maika
Hind:	Bel
Mal:	Koovalam
Pers:	Shol
Tam:	Vilvam
Tel:	Bilvamu

DESCRIPTION:

Leaves

Leaves commonly alternate and 3- foliate arrangement of the leaves, in some of variants rarely 5- foliate arrangements. Petiole is 2.5-6.3 cm long, terete. Dimension of the leaflets 5-10 by 2.5-6.3cm. The sizes of leaflets are petiole length varies in different variants. In T.S., Petiole is broad 'C' shaped in outline with a single layer of schizogenous cavity and a conspicuous broad. 'C' shaped vascular bundle in the centre. Epidermis is single layered occasionally interrupted with sunken stomata on both surfaces and over- lined by a thick layer of cuticle. Interior to the epidermis is a many layered palisade tissue, which consists of closely, packed oval cell without much intercellular space. The chloroplasts are more abundant in the palisade cells and less in the spongy tissue. Both upper and lower epidermal layers bear stomata. Each stoma has two guard cells and two subsidiary cells and they correspond to rubiaceous type. The numerical values like vein- islet number, palisade ratio and stomata index are significantly diagnostic features of this species (Krishnan Nambiar *et al.*, 2000).

Stem bark:

Grey in color more warty and less number of cracks and fissures. Thickness 4-8mm; cork zone showing 5-8 stratification. Stone cells present in more number of groups in the phelloderm and also present in groups in the phloem fibers present in groups arranged in concentric rings. Transverse section of the stem reveals the presence of 1) Well developed periderm consisting of cork, phellogen and phelloderm, 2) Distinct patches of stone cells

above the phloem region, 3) Several layers of cambium, 4) Conspicuous xylem with large vessels and uniseriate medullary rays and 5) Parenchymatous pith.(Anonymous, 1976).

Fruits:

Fruit, sub-globous, 5-18cm in diameter, externally greenish when young, yellowish brown when ripe, rind about 1.5mm-3mm thick hard and woody, surface smooth or slightly granular bearing a circular scars at the point of attachment with peduncle.

Root:

. Outer zone of cork which gets peeled off consequent on secondary growth. This is followed by phellogen and secondary cortex whose cells contain abundance of starch grains. Interior to the cortex is the characteristic concentric patches of sclerenchyma. Phloem is concentrically arranged; phloem cells alternating with narrow strip of sclerenchyma. Medullary rays, distinct ring of cambium, wood consisting of large vessels, tracheids and fibers, uniseriate and biseriate medullary rays filled with starch grains, and pentarch primary xylem are other features (Krishnan Nambiar *et al*., 2000).

ETHNOMEDICINAL USES

LEAVES:

George *et al*., 2003, had reviewed *Aegle marmelos L* used in the treatment of backache, abdominal disorder, vomiting, cut& wounds, dropsy, cholera, diarrhea, cardio tonic, blood sugar, hair tonic.

Gaur, 1999, had reported the *Aegle marmelos* linn used in the treatment of killing intestinal worms, stimulation of respiratory contraction.

Kirtiker K.R, *et al.,* 1935, had reported *Aegle marmelos* linn used in the treatment of Anti-diabetic agent in local people Maharashtra.

Folklore of Assam used the *Aegle marmelos* linn in the treatment of vomiting, backache

Tribal ‘of Ranchi (dt) of Bihar used *Aegle marmelos* linn in the treatment of jaundice, abdominal heat.

Saxena A.P, *et al*., 1981, had reported *Aegle marmelos* linn used in the treatment of killing of intestinal worms, stimulation of respiratory contraction.

Phytochemical review:

Leaves:

Amirtham S., 2016 had investigated preliminary phytochemical screening of different (benzene, chloroform) extract of aegle marmelos linn. Benzene extracts showed the presence of alkaloid, emodin, phenolics, volatile, and absence of flavonoid, triterpines, steroids,glycoside,,xanthoprotein. Chloroform extract showed the presence of xanthoprotein, carbohydrate, volatile oil, phenolics, emodin, alkaloid, and absence of flavonoid,and anthracene glycoside.

Sonu Singh and Neeta Singh., 2016; had investigated preliminary phytochemical screening on chloroform extract of aegle marmelos linn. Showed the presence of Alkaloids, Amino acids, Anthocyanin, Carbohydrates, Cardiac Glycosides, Coumarins, Diterpenes, Emodins, Fatty acids, Flavonoids, glycosides, Phlobatannin, , Proteins, Phenols, Saponin, , Tannin, Terpenoids and absence of Leucoanthocyanin, Phytosterols,, Steroids

Sugeng *et al*., 2001; had isolate the two alkaloid4,7,8 –trimethoxyfuroquinoline (skimmianine) and aegeline from aegle marmelos linn. It's confirmed by spectrometric method.

Nadeem *et* al 2010 ; had investigated the total phenolic and total flavonoid content using folin ciocatteu reagent in different part of aegle marmelos linn .the leaves contain larger amount of total phenolic and total flavonoid content compared other parts of the plant.

Yadav N.P., 2009; had reported the limonene (82.4%) is the major constituent of aegle marmelos linn.

Kothari,S., *et al.*, 2011; studied the preliminary phytochemical screening on different extract (ether, methanol, chloroform.) aegle marmelos linn. Methanol extract showed the presence of flavonoid. Phenolics, tannins, saponins, coumarin, sterols terpenoids, and the ether ,and chloroform extract showed only presence of sterols and phenols.

Murugiah and, venkatachalam R., 2015; had isolated the aegeline from ethanol extract of aegle marmelos linn using column chromatographic method.

Amit pandey and Rashmi, Mishra., 2011; studied the preliminary phytochemical screening on different extract (ethanol, methanol, water, ethyl acetate) aegle marmelos linn . it showed the presence of tannins, sapponoins, terpenoids, alkaloids,polyphenol.

Arunachalam D, *et al*., 2012 ; had study the preliminary phytochemical screening on methanolic extract of *aegle marmelos* linn.it showed the presence of amino acid terpenoid, steroid., alkaloid , flavonoid and absence antharacene glycoside, cardiac glycoside.

Ariharan V.N ,Nagendra Prasad .P. ,2014.; had study the preliminary phytochemical screening on chloroform extract of aegle marmelos linn.it showed the presence of flavonoid, phenol, terpenoids and alkaloid, amino acid, coumarin ,and absence steroids, phytosterols.

Phuwapraisirisan *et al.,* (2008) ;have isolated a series of phenylethyl cinnamides from the leaves of aegle marmelos linn.

Maity,P ,*et al* .,2009; report the leaves aegle marmelos contain tannins, limonene, aegline, cineole, skimmianine.

Manandhar , M.D , *et al*., 1978; report the actve principle of o-(3,3- dimethylallyl) – halofordinol in aegle marmelos linn leaves.

Nandkarni,A.K ,*et al* .,1976; report the marmelosin a active constituent in aegle marmelos linn leaves.

Sharma,B.R., et al., 1980 report the aegle marmelos linn leaves contain marmesinnin, rutin, beta sistosterol, and marmeline.

Arul V, *et al*., 2004; report the aegle marmelos linn leaves contain umbelliferone.

Farooq S., 2005; report the aegle marmelos linn leaves contain flavones, lupeol, citral, eugenol, and citronellal.

Ali *et al* ., 2004; report the 7-geranyloxycoumarin named marmenol isolated from the methanolic extract of aegle marmelos linn .the structure of marmenol were established with the help of NMR spectroscopy.

PHARMACOLOGICAL ACTIVITY:

ANTI-MICROBIAL ACTIVITY:

Sivaraj R, *et al*., 2011; studied the antimicrobial activity of the leaves of *A. marmelos* was performed by agar well diffusion method. The aqueous, petroleum ether and ethanol extract of the leaves of *Aegle marmelos* exhibited efficient antimicrobial activity against *Escherichia coli*, *Streptococcus pneumoniae, Salmonella typhi*, *Klebsiella pneumoniae* and *Proteus vulgaris.* The ethanolic extract shows activity against *Penicillium chrysogenum* and the petroleum ether and aqueous extract shows activity against *Fusarium oxysporum.*

Gavimath C.C, *et al* .,2011; the antimicrobial activity was checked by disc diffusion method. The petroleum ether extract of leaves was checked against multi resistant strains of *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Salmonella typhi, Proteus vulgaris, Pseudomonas aeruginosa* and *Klebsiella pneumoniae.*The extract showed antimicrobial activity against gram-negative strains was higher than that of gram positive strains.

ANTI-DIABETIC ACTIVITY:

Kuttan ,R.,and Sabu., MC ., 2004; studied on leaf extract of Aegle Marmelos on Alloxan induced diabetes and reported that used extract was enough capable to reduce oxidative stress by scavenging lipid peroxidation and enhancing certain Anti-oxidant levels which causes lowering of elevated blood glucose level.

Sevugan , A, *et al.,* 2008; the leaf and callus extracts possess the ability to stimulate the insulin secreting cells of pancreas. Among the various extracts used, the methanol extracts of the leaf and callus revealed the maximum anti-diabetic effect. The results suggested that both the callus and leaf materials contain anti-diabetic active principles, which reduced the sugar level in STZ-diabetic rabbits.

ANALGESIC ACTIVITY:

Shankaranath , V, *et al.,* 2007; studied the methanol extract of leaves of Aegle marmelos at a dose level of 200 and 300 mg/kg showed significant analgesic activity on acetic acid-induced writhing and tail flick test in mice.

ANTIFUNGAL ACTIVITY:

Rana B.K, *et al* .,1997;studied the antifungal activity of essential oil isolated from the leaves of bael (Aegle marmelos (L.) has been evaluate using spore germination assay. The oil exhibited variable efficacy against different fungal isolates and 100% inhibition of spore germination of all the fungi tested was observed at500 ppm. However, the most resistant fungus, Fusariumudum was inhibited 80% at 400ppm. Kinetic studies showed concentration as well as time dependant complex inhibition of spore germination by essential oil.

Balakumar S , *et al.,* 2011;studied the antifungal activity of the leaves of *Aegle marmelos* was reported against clinical isolates of dermatophytes. *A. marmelos* leaf extracts and fractions were found to have fungicidal activity against *Trichophyton mentagrophytes, T.rubrum, Microsporum canis, M. gypseum,Epidermophyton floccosum.*.

ANTIFERTILITY ACTIVITY:

Chauhan A ,*et.al* .,2007; studied the ethanolic extract from the leaves of A. marmelos. on the reproductive system of male albino rats was investigated at three different doses ,namely, 100, 200 and 300 mg−1 kg −1 day−1 for each rat for 60 days. Recovery was also investigated after withdrawal of 120 days. All the major accessory sex organs shed weight post administration of the extract .There was a marked reduction in motility and density of the sperm derived from cauda epididymis of the treated animals. A. marmelos reduced fertility of male rats by100% at the 300-mg dose level. Serum testosterone level also decreased significantly in all the experimental groups. The leaf extract of A. marmelos suppresses fertility in male rats .Complete recovery of fertility was observed following the withdrawal of drug. Absence of any deleterious effect on the vital organs points to the safe use of the extract.

ANTI-OXIDANT ACTIVITY:

Vanitha P Reddy , *et al* .,2013 ;reported Aegle marmelos (AM) leaves were extracted with methanol (ME), ethanol (EE), water (WE) and analyzed for antioxidant activities by DPPH radical scavenging method, reducing power and in vitro inhibition by Fenton's reagent-induced oxidation of lipid system. The three extracts showed varying degree of efficacy in each assay in a dose dependent manner. The inhibition of MDA formation in Linseed oil by EE (47%) was significantly ($P < 0.05$) higher than WE (28%) and ME (23%) but less than α-Tocopherol (80%). WE showed maximum stability to high temperature. The antioxidant activity of EE at pH 4 was significantly higher ($P < 0.05$) compared with WE and ME. At pH 7, the antioxidant activity of all the three extracts remained unchanged. Data indicates that potential exists for the utilization of Aegle marmelos as a natural antioxidant.

CYTOTOXICITY:

Rajbir Bhatti, *et al.*, 2013; evaluate the anti-proliferative activity of the leaf extracts Aegle marmelos (L.) Correa (Rutaceae) and the chromatographic fractions of the most active extract. and assessed using human cancer cell lines of lung (A-549), colon (CoLo-05), ovary (IGR-OV-1) ,prostrate (PC3), leukemia (THP-1) and breast (MCF-7) cancer. Bioactivity-derived fractionation was carried out for most active extract by column chromatography. The phytochemical studies indicated alkaloids, anthraquinones, terpenoids in the alcohol, chloroform extracts and tannins, terpenoids, reducing sugars in the petroleum ether and hexane extracts. Ethanol extract showed maximum inhibition in colon and breast carcinoma cell lines at a dose of 100μg/ml. the different extracts investigated, ethanol extract exhibited significant antiproliferative activity and the fraction containing furanocoumarin, imperatorin showed antiproliferative activity against leukemia cell line with IC50 of 12.5 μg/ml.

RADIO PROTECTIVE:

G C Jagetia, *et al*., 2004; investigate the radio protective activity of a leaf extract of leaf (Aegle marmelos) (AME) in mice exposed to different doses of gamma-radiation. The acute toxicity of AME was evaluated in Swiss albino male mice administered various doses of AME. For radioprotection studies, mice were administered different doses, 0, 5, 10, 15, 20 or 40 mg kg(-1), of AME or sterile physiological saline intraperitoneally once daily consecutively for 5 days before exposure to 10 Gy 60Co gamma-radiation or five doses of 15 mg kg(-1) AME before exposure to 6, 7, 8, 9, 10 or 11 Gy. AME treatment reduced the symptoms of radiation-induced sickness and increased survival. The radio protective action might be due to free-radical scavenging and arrest of lipid peroxidation accompanied by an elevation in glutathione.

STEM BARK:

ETHANOMEDICAL USE:

Ritu Gupta., 2016; reported the bark is used for curing of diarrhoea in tribals of andhrapradesh people.

Joshi P., 1986; reported the bark used as fish poison in tribals of southern Rajasthan.

PHYTO CHEMICAL REVIEW:

Chatterjee,A and Mitra,SS., 1949; report the fagrine and marmin isolated from the stem bark of aegle marmelos.

Chatterjee *et al.,* 1978; report the Sterols and triterpenoids such as lupeol and β-sitosterol, α and β-amyrin, flavonoids such as rutin and coumarins have been isolated from the stem-bark of the plant.

Maity,P., *et al*., 2009; report the aegle marmelos stem bark contain skimmainine.

PHARMACOLOGICAL REVIEW:

ANTI-DIABETIC ACTIVITY:

Vikas Kumar , *et al* .,2013; evaluate the anti-diabetic activity in stem bark of Aegle marmelos Correa. in STZ (streptozotocin) induced diabetic rat. Diabetes was induced in rat by single intraperitoneal injection of STZ (60 mg/kg). Diabetes was measured by change the level blood glucose, plasma insulin. Administration of different dose of UFG for 28 days showed significantly ($P < 0.001$) decreased in Fasting blood glucose level and improve plasma insulin level as compared to the diabetic control group.

Gopalsamy Rajiv Gandhi, et *al., 2012*; studied the anti-diabetic potential of Aegle marmelos (L.) Corr. (Rutaceae) bark in a diabetic rat model. Dose dependent effects of methanol extract of Aegle marmelos bark (AM) (200 and 400 mg/kg) on blood glucose, plasma insulin, glycated hemoglobin (HbA1c), total protein, hepatic glycogen, marker enzymes of hepatic function and carbohydrate metabolism were evaluated in (streptozotocin) STZ-induced diabetic rats by oral administration for 30 days. Structural integrity of pancreatic islets was assessed by routine histology while, their functional status was assessed by immunolocalization for insulin AM at 200 and 400 mg/kg showed significant reduction in blood glucose level by 19.14% and 47.32%, respectively in diabetic rats. AM treatment significantly increased insulin level, and produced similar effects on other biochemical parameters. Histological studies showed the regenerative effect of AM on the β-cells of diabetic rats.

ANTI-FERTILITY ACTIVITY:

Shyam S Agarwal, *et al.*, 2012; evaluate the methanolic bark extracts of Aegle marmelos (L.) r male antifertility activity on albino wistar rats. Methanolic bark extract of Aegle marmelos at the dose of 200, 400, and 600 mg/Kg b.w was administered orally for 60 days. Lonidamine was used as standard drug to compare the effect of extract. Sperm analysis results showed reduction in sperm density, motility, viability and sperm acrosomal integrity without interfering libido and vital organ body weight. . Aegle marmelos barks methanolic extract as strong candidate for male contraceptive via its ability to produce complete inhibition of pregnancy.

FRUIT:

ETHANOMEDICAL USE:

Tribal of rural area of Jammu & Kashmir used the *Aegle marmelos* linn in the treatment of laxative.

In kokan region small unripe fruits used in the treatment of piles.

Parmar,C and Kaushal,MK , 1982; had literaturely reviwed the traditional healers of southern Chhattisgarh used the *Aegle marmelos* linn in the treatment of burn cases.

Veerapan ,AK *et al* .,2000; had reviewed *Aegle marmelos* linn used in the treatment of antiviral, gonorrhea, epilepsy.

PHYTO REVIEW:

Sharma , *et al*., 1980; report the aegle marmelos linn fruit contain alloimperatorin, imperatorin, and scopoletin.

Kakiuchi,N, *et al*., 1991; report the aegle marmelos linn fruit contain auraptene.

Maity,P *et al*., 2009;report the aegle marmelos linn fruit contain calcium compound, linoleic acid.

Barthkumar,NN and Arnold ,NP .,1989; report the aegle marmelos linn fruit contain glutamic acid , glycine , lysine , phenylalanine, proline , skimming , umbelliferone , xanthotoxol.

Badam,L. *et al*., 2002; report the aegle marmelos linn fruit contain marmelosine.

Chakthong,S., *et al*., 2012; report the aegle marmelos contain psoralen.

Farooq,S., 2005; report the aegle marmelos contain luvangetin, marmelide, tannins.

Rattan, K ,*et al* .,1981; report the homogenous, neutral polysaccharide isolated from the fruit pulp of aegle marmelos. Contain arabinose, galactose, and glucose.

PHARMACOLOGICAL REVIEW:

ANTI-DIABETIC ACTIVITY:

Kamala Kannan N., 2003; investigated the hypoglycemic effect of the water extract of the fruits of *Aegle marmelos* was examined in streptozotocin-induced diabetic Wistar rats. Oral administration of the water extract (125 and 250 mgkg−1) twice a day for 4 weeks resulted in significant reductions in blood glucose, plasma thiobarbituric acid reactive substances, hydro peroxides, ceruloplasmin and_-tocopherol and a significant elevation in plasma reduced glutathione and Vitamin C in diabetic rats. The effect of the extract at a dose of 250 mg kg−1 was more effective than glibenclamide in restoring the values of these parameters. The results of this study clearly showed the hypoglycemic activity of the fruit extract.

ANTI MICROBIAL ACTIVITY:

Maheshwari,VL. *et al.,* 2009 ;studied on ethnolic extract of dried fruit pulp of Aegle Marmelos against various intestinal pathogens i.e. Shigella boydii, S. sonnei & S. Flexneri. the extract were effective against all.

RADIO PROTECTIVE ACTIVITY:

Ganesh Chandra Jagetia., 2006 ;investigate the radio protective effect of a hydro alcoholic extracted material from the fruit of Aegle marmelos (AME) studied in mice exposed to different doses of gamma radiation. the acute toxicity study of AME showed that it was nontoxic up to a dose of 6 g/kg body weight, the highest drug dose that could be administered. Irradiation of animals resulted in a dose-dependent elevation in lipid peroxidation in liver, kidney, stomach, and intestine of mice. Conversely, GSH concentration declined in a dose-dependent manner. Treatment of animals with AME before irradiation caused a significant decrease in the lipid peroxidation accompanied by a significant elevation in the GSH concentration in liver, kidney, stomach, and intestine of mice determined at 31 days post irradiation.

ANTI-GENOTOXICITY:

Kaur p ,*et al* ., 2009; report the Methanol and acetone extract of dried fruit were used to evaluate antigenotoxic activity of *Aegle marmelos* in Human Blood Lymphocytes and E. coli PQ. It was found that both methanol extract and acetone extract were quite effective in decreasing the SOS response induced by hydrogen peroxide and aflatoxin B1 in the SOS chromotest.

ANTI-ELASTASE ACTIVITY:

Vignesh Sundararajan, *et al.,* 2018; investigated the anti-aging properties and cytotoxicity in vitro individually as well as in a poly herbal formulation containing the four plant extracts in different ratios. The phytochemical contents of the plant extracts were investigated for radical scavenging activity and total reducing power. Based upon its anti-oxidant properties, a poly herbal formulation containing unripe and ripe fruit pulp of Aegle marmelos, Poly Herbal Formulation due to better anti-oxidant and anti-elastase activities in NIH3T3 fibroblast cells. Based on these results these beneficial plant extracts were identified for its potential application as an anti-aging agent in skin creams as well as an anti-proliferation compound against cancer cells.

INFLAMMATORY BOWEL DISEASES:

Behera, J, *et al*., 2012; evaluate the effect of Aegle marmelos unripe fruit extract (AMFE) on inflammatory bowel disease (IBD) in Wistar albino rats. acetic acid induced ulcerative colitis (1 ml of 4% acetic acid solution, trans rectal) and indomethacin-induced enter colitis (10 mg/kg, single dose,) The extract was administered orally at different dose of 150, 200 and 250 mg/kg body weight. Disease pathogenesis was assessed by measuring disease activity index (DAI), macroscopic score, microscopic score, mesenteric mast cell protection, superoxide dismutase (SOD), and malonaldehyde (MDA) levels in the above two models .The results showed a dose dependent decrease in intestinal inflammation following treatment with AMFE.

COLITIS:

Gautam, M.K.,*et al* .,2013;studied the curative effect of 50% ethanol extract of dried fruit pulp of AM (AME) against 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced experimental colitis. AME showed antibacterial activity against intestinal pathogens and decreased colonic mucosal damage and inflammation, diarrhea, colonic free radicals and MPO and enhanced body weight and colonic antioxidants level affected by TNBS. The effects of AME on the above parameters were comparable with sulfasalazine, a known colitis protective drug(100 mg/kg, oral). AME shows curative effects against TNBS-induced colitis by its antibacterial activity.

ROOT:

ETHANOMEDICAL USE:

Tribal of Orissa (bhottada) used *Aegle marmelos* linn in the treatment of mad dog bite.

Tribal of Andhra Pradesh used *Aegle marmelos* linn in the treatment of bone fracture.

Tribal of Malabar cost people used *Aegle marmelos* linn in the treatment of hypochondrias, palpitation of heart.

Ohashi, *et al.*, 1995; had literature reviewed *Aegle marmelos* linn *used* in the treatment of hypoglycemic.

Veerapan, *et al,. 2000;* had reviewed *Aegle marmelos* linn *used* in the treatment of gastric troubles, heart disorder, rhematatism anti-amoebic, dog bite.

PHYTOCHEMICAL REVIEW:

Surat laphookhieo, *et al.,* 2011; reported the new natural oxazoline derivative named aeglemarmelosine and 8 known compounds isolated from the aegle marmelos root and twigs. Compound 1-6 were isolated from the root and 7-9 were obtained from the twigs

Shoeb,A, *et al*., 1973; report the *aegle marmelos* root contain A-methylscpoletin, skimming, scpoletin, timbamine.

Basu,D and Sen,R., 1974; report the aegle marmelos root contain psoralen, umbelliferone, xanthotoxin.

Chatterjee *et al.*, 1978; report the Sterols and triterpenoids such as lupeol and β-sitosterol, α and β-amyrin, flavonoids such as rutin and coumarins have been isolated from the root.

PHARMACOLOGICAL REVIEW:

ANTI – DIARRHEAL ACTIVITY:

Mazumder, R, *et al* .,2006; report the chloroform extract *Aegle marmelos* linn root anti-diarrheal activity in vitro (disc diffusion) , and in vivo method studied in rat animal model.in in vitro method the extract active against strain of vibrio cholera , followed by E.coli and shigella spp.in animal model have significant activity in castor oil induced .

ANTI INFLAMMATORY:

Jyoti M Benni., 2011; evaluate the anti-inflammatory activity of the aqueous root extract of Aegle marmelos in experimental acute and chronic inflammatory animal models. Aqueous extract of root of AM was prepared and tested for anti-inflammatory activity in albino rats weighing 150-280 grams. The animals were randomly divided into 3 groups of 6 each; one group served as control and other two groups received indomethacin and AM orally 1 hour prior to experimentation. The in vivo anti-inflammatory activity was studied using the acute (Carrageenan induced paw edema) and chronic (Cotton pellet induced granuloma) animal models. Aegle marmelos showed highly significant activity in acute model and but a trend of anti-inflammatory activity in chronic model studied.

CHAPTER-3

AIM AND OBJECTIVE

CHAPTER -3

AIM AND SCOPE OF THE PRESENT STUDY

The awareness of drugs goes back to prehistoric times. Man as savage known by experience how to relieve his sufferings by the use of herbs growing him. In recent times, focus on plant research has increased all over the world and a large body of evidence has collected to show immense potential of medicinal plants used in various traditional systems.

This century has witnessed many great achievements in the field of medicine. One of the great advancement in medical care in this century has been the introduction of a number of drugs which are having more toxic side effects. The floras of India are both abundant and rich in every kind and description of plant well known. In country, the traditional medicine has survived and is in practice in our rural areas. Nearly 76% of our population can offer this form of medicine. This is also prevailing situation in all the developing countries taking note on the realities of these situations because of their effectiveness, less side effects and relatively low cost

.

The plants *Aegle marmelos* has been appealed to acquire a diversity of therapeutic properties. These plants are having large potential to treat the disorders like cholesterol, diarrhoea, diabetes, inflammation, peptic ulcer and dysentery, which as well has the property of anticancer, anticonvulsant, anti-bacterial, cardio protective, radio protective, antioxidant, analgesic, anti-pyretic, anti-fungal, constipation, respiratory infection, hepatoprotective, and wound healing etc.

After the ethno medical information and folk claims it is observed that the plants *Aegle marmelos* medicinal properties linked to toxicity studies, anti-microbial, anticancer, anti-oxidant, anti-inflammatory and anti-convulsant which have not been scientifically validated and only some of the phytochemical studies have been carried out and reported for the presence of Steroids, Alkaloids, Flavonoids, Tannins, Saponins. Therefore, the present investigation is concerned with the widely distributed indigenous medicinal plants *Aegle marmelos.*

Beginning the survey and appropriate literature of the plants *Aegle marmelos* is the most well known in traditional medicine practices and the diverse medicinal property attributed to inspire us to investigate the photo protective activity.

Aim of the study is IDIOSYNCRATIC APPORCH FOR AN ERRATIC AND STUNNING MANAGEMENT OF *Aegle marmelos* linn LEAVES ON PHOTOSENSITIVITY BY USING ZEBRAFISH EMBRYO MODEL.

OBJECTIVE:

The objective of the study divided into three parts.

PART 1: PHARMACOGNOSTICAL STUDY:

- Collection and authentication of plant
- Morphological study of the plant
- Microscopial study of the leaves
  - ✓ Anatomical study
  - ✓ Powder microscopy
  - ✓ Quantative Microscopy
- Physiochemical parameters
  - ✓ Ash value
  - ✓ Loss on drying
  - ✓ Extractive value

PART 2: PRELIMINARY PHYTOCHEMICAL SCREENING:

- Qualitative analysis of the leaves for the presence of various phyto constituents
- Determination of total flavonoid , phenolic , tannin , coumarin content
- To study the TLC profile of ethanolic extract of *Aegle marmelos* linn leaves.
- Isolation of bioactive component from ethanolic extract of aegle marmelos by using coloumn chromatography
- Identification and characterization of isolated compound by using

- ✓ Chemical test
- ✓ Thin layer chromatography
- ✓ Spectral studies

PART 3: PHARMACOLOGICAL STUDIES:

- ❖ In vitro anti-oxidant activity
  - ✓ Total antioxidant activity by Phosphomolybdenum Method.
  - ✓ Reducing power assay
- ❖ Toxicological studies of EEAM on the early development of zebra fish
  - ✓ Embryo toxicity study
- ❖ In vitro sun protection factor determined by UV -Visble spectroscopy method.
- ❖ In vivo photo protective activity against UVA radiation using zebra fish embryo model.

CHAPTER-4

MATERIAL AND METHOD

CHAPTER-4

MATERIALS AND METHOD:

Nowadays there is a renewed interest in drugs of natural origin simply, because they were consider as green medicine and is always supposed to be safe. Another factor is the incidences of harmful nature of synthetic drugs, which were regarded as harmful to human beings and environment. The advantage of natural drugs is their easy availability, economic and less or no side effects but the disadvantage is that they are the victims of adulteration. The more effective the natural drug more is its demand and the chances of non-availability increases. To meet the growing demand, the natural drug is easily adulterated with low-grade material.

Therapeutic efficacy of medicinal plants depends upon the quality and quantity of chemical constituents. The misuse of herbal medicine or natural products starts with wrong identification. The most common error is one common vernacular name is given to two or more entirely different species **(**Dinesh Kumar C, 2007). All these problems can be solved by pharmacognostical studies of medicinal plants. It is very important and in fact essential to laydown pharmacognostic specifications of medicinal plants which are used in various drugs.

Pharmacognosy is the study of medicines derived from natural sources, mainly from plants. It deals with standardization, authentication and study of natural drugs. Most of the research in pharmacognosy has been done in identifying controversial species of plants, authentication of commonly used traditional medicinal plants through morphological, phytochemical and physicochemical analysis. The importance of pharmacognosy has been widely felt in recent times. Unlike taxonomic identification, Pharmacognostical study

includes physiochemical parameters which help in identifying adulteration in dry powder form also. This is again necessary because once the plant is dried and made into powder form, it loses its morphological identity and easily prone to adulteration. Pharmacognostic studies ensure plant identity, standardization parameters, which will help and prevents adulterations. Such studies will help in authentication of the plants and ensures reproducible quality of herbal products, which will lead to safety, and efficacy of natural products. The Pharmacognostical standardization parameters that were generally described below.

After decades of serious obsession with the modern medicinal system, people have started looking at the ancient healing systems like Ayurveda, Siddha and Unani to treat the various types of ailments. This is because of the adverse effects associated with synthetic drugs. Herbal traditional medicines have gained considerable momentum worldwide during the past decade and play a paramount role in health care programs especially in developing countries.

PHARMACOGNOSTICAL STUDIES:

Collection and identification of Plant Materials:

The leaves of *Aegle marmelos Linn* were collected from Nagapattinam, Tamilnadu India, during the months of October and November 2017 and all the primary work done (washing, drying…etc.).The plant materials were identified and authenticated by Dr.D.Stephen, Asst.professor in botany, The American College, Madurai-20. The collected plant Material was free form disease and also free from contamination of other plants. .

MACROSCOPIAL EVALUATION:

Organoleptic evaluation can be done, by means of sense organs, which provide the simplest as well as quickest means to establish the identity and purity to ensure quality of a particular drug. Organoleptic characters (External appearance) such as shape, size, colour, Odour, taste and leaf structure like margin, apex, base, surface, venation and inflorescence, etc. are evaluated.(Siddiqui and Hakim,MA.,19950 The macroscopic features observed for the leaves of the plant are presented in **Table. 1 and plate 1 to 3**

MICROSCOPIAL EVALUATION:

Sectioning:

The paraffin-embedded specimen was sectioned with the help of rotary microtome. The thickness of the sections was 10-12µm, de-waxing of the sections were carried out by customary procedure (Johansen, 1940). The sections were stained with toludineblue as per the method published by O'Brien *et al.* (1964). Since toludine blue is a polychromatic stain, the staining results were remarkably good, and some cytochemical reactions were obtained. The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc. The sections were, also stained with saffranin and fast green and iodine wherever necessary.

Photomicrographs:

Photographs of different magnifications were taken with Nikon lab photo 2 microscopic units. For normal observation bright field was used. For the study of crystals, starch grains and lignified cells, polarized light was employed. Since these structures have bierfringiest property, under polarized light they appear bright against dark background. The

scale bars on the photomicrographs indicated the magnification of the figures. The microscopic features observed for the leaves of the plant are presented in **plate .4 to 8**

QUANTITATIVE MEASUREMENTS :(Kokate, CK 1994)

DETERMINATION OF LEAF CONSTANTS:

The stomatal number, stomatal index, vein islet number and vein termination number were determined on fresh leaves by using standard procedures.

Determination of Stomatal Number:

The **stomatal number** may be defined as the "average number of stomata per square mm area of epidermis of the leaf".

Small pieces of upper and lower epidermal peelings of the leaves were mounted onto a slide. The camera Lucida and stage micrometer were used to draw 1mm square on a paper. The stage micrometer was replaced by the preparation slide. The stomata were marked in that unit area were observed under microscope and. The number of stomata present in unit area was calculated. Ten such readings were taken and the average of stomatal number was calculated and presented in the **Table 2** for both upper and lower epidermis.

Determination of Stomatal Index:

The **stomatal index** is the percentage of the ratio of the numbers of stomata to the total number of epidermal cells where each stoma also being counted as one cell. It is calculated using the following formula **S.I = ×100; w**here S is the number of stomata per unit area and E is the number of epidermal cells in the same unit area

The procedure adopted for the determination of stomatal number was followed and the preparation was observed under high power. The epidermal cells and the stomata were counted. From these values the stomatal index was calculated using the above

formula and was given in **Table .2**

Determination of vein islet number and vein termination number

The term vein islet is used to denote the minute area of photo synthetic tissue encircled by the ultimate division of the conducting strands. The **vein islet number** may be defined as the" number of vein islets per square mm area".

The term **vein termination number** may be defined as the "number of vein terminations present in one square mm area of the photosynthetic tissue".

Small pieces of leaves were cut on the lamina between midrib and the margin, cleared in chloral hydrate and mounted on a slide. The camera Lucida and drawing board were arranged. With the help of a stage micrometer, camera Lucida and microscope, 1mm square was drawn on the paper. Then the stage micrometer was replaced by the sample slides and the veins were traced over the square. The vein islets and vein terminations were counted in the square. Ten such readings were taken, the average was calculated, and the results were presented in **Table.2**

Physiochemical Evaluation of *Aegle marmelos* linn*:*

Preparation of Powder:

The collected whole plant samples were washed thoroughly with water to free from foreign organic matters. Then the plant materials were cut in to small pieces and dried for few days. These dried whole materials were pulverized mechanically and passed through 40 mess sieve to obtain coarse powder and store in an air tight container. These coarse powdered materials were used for further Pharmacognostical physiochemical phytochemical, fluorescent analysis and preparation of extracts for pharmacological evaluation.

Determination of Colour:

The untreated part of the drug was taken and colour of the drug was examined under sunlight.

Determination of Odor:

A small portion of the drug was taken, slowly and repeatedly inhaled the air over the material and examined the odor

Determination of Taste:

For taste, a small portion of drug was taken on the tongue and find out the taste of drug.

The parameters which are studied are moisture content, loss on drying, total ash, acid-insoluble ash, alcohol and water-soluble extractive values, petroleum ether soluble extractive value, ethyl acetate soluble extractive value, acetone soluble extractive value, etc.

The residue remaining/left after incineration of the crude drug is designated as ash. The ash remaining following the ignition of medicinal plants is determined by three different

methods which measures, total ash, acid-insoluble and water soluble ash .Ash values are used to determine quality and purity of crude drug. It indicates presence of various impurities like carbonate, oxalate and silicate. The water soluble ash is used to estimate the amount of inorganic compound present in drugs. The acid insoluble ash consist mainly silica and indicate contamination with earth material. Moisture content of drugs should be at minimal level to discourage the growth of bacteria, yeast or fungi during storage. Estimation of extractive values determines the amount of the active constituents in a given amount of plant material when extracted with a particular solvent. The extractions of any crude drug with a particular solvent yield a solution containing different phytoconstituents. The compositions of these phytoconstituents depend upon the nature of the drug and the solvent used. It also gives an indication whether the crude drug is exhausted or not. **[**Bele A. A, et al.2011**]**.

Determination of Total Ash

About 2 g of the powdered drug was accurately weighed in silica crucible, which was previously ignited and weighed. The powdered drug was spread as a fine even layer on the bottom of the crucible. The crucible was incinerated at a temperature not exeeding450°C, until free from carbon. The procedure was repeated to get the constant weight. The percentage of the total ash was calculated with reference to the air-dried drug and the values are recorded in Table-

Determination of water soluble ash

The ash obtained as described in the determination of total ash was boiled for five minutes with 25 ml of water. The insoluble matter was collected on an ash less filter paper

and washed with hot water. The insoluble ash was transferred into a pre-weighed silica crucible and ignited for 15 minutes at a temperature not exceeding 450°C.

The procedure was repeated to get constant weight. The weight of insoluble matter was subtracted from the weight of total ash. The difference in weight was considered as water soluble ash. The percentage of water soluble ash was calculated with reference to the air-dried drug .**Table .3**

Determination of Acid soluble Ash

The ash obtained as described in the determination of total ash was boiled with 25 ml of 2M Hydrochloric acid for 5 minutes. The insoluble ash was collected on an ash less filter paper and was washed with hot water. The insoluble ash was transferred into a pre weighed silica crucible, was ignited, cooled in desiccator and weighed. The procedure was repeated to get constant weight. The percentage of acid insoluble ash was calculated with reference to air-dried drug **Table.3**

Extractive Value (Kokate, 1994)

Extractive value of crude drug is useful for the evaluation especially when the constituent of a drug cannot be readily estimated by any other means. Further these values are the indicatives of the approximate measures of their chemical constituents and the nature of the constituent present in the crude drug. Taking into consideration the diversity in chemical nature and the properties of content of drugs, various solvents are used for determination of extractives. The solvent used for extraction is in a position to dissolve appreciable quantities of substance desired.

Determination of Extractive values

Petroleum ether soluble extractive value

An accurately weighed 5g of coarsely powdered air dried drug was macerated in 100mL of petroleum ether in a closed flask for 24h, shaking frequently during 6h and allowed to stand for 18h. It was filtered rapidly taking precautions against loss of solvent. 25mL of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish and dried at 105˚C to constant weight. The percentage of the petroleum ether soluble extractive with reference to the air dried drug was calculated and presented in **table 3.**

Ethanol soluble extractive

An accurately weighed 5g of coarsely powdered air dried drug was macerated in 100mL of ethanol in a closed flask for 24h, shaking frequently during 6h and allowed to stand for 18h. It was filtered rapidly taking precautions against loss of solvent. 25mL of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish and dried at 105˚C to constant weight. The percentage of the petroleum ether soluble extractive with reference to the air dried drug was calculated and presented in **table 3.**

Water-Soluble Extractive

An accurately weighed 5g of coarsely powdered air dried drug was macerated in 100mL of chloroform water in a closed flask for 24h, shaking frequently during 6h and allowed to stand for 18h. It was filtered rapidly taking precautions against loss of solvent. 25mL of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish and

dried at 105˚C to constant weight. The percentage of the petroleum ether soluble extractive with reference to the air dried drug was calculated and presented in **table.**

Determination of chloroform soluble extractives

The procedure followed for the determination of alcohol soluble extractive value was adopted for the determination of chloroform soluble extractive, ethyl acetate soluble extractive, and benzene soluble extractive. Instead of ethanol respective solvents were used for the determination of their extractive values.

The percentage of chloroform soluble extractives were calculated and presented in **table.3**

Loss on Drying

It is used for determination of moisture content. The percentage of active chemical constituents in crude drugs is mentioned on air-dried basis. Hence, the moisture content of the drug should be determined and should also be controlled. The moisture content of a drug should be minimized in order to prevent decomposition of crude drugs either due to chemical change or microbial contamination.

About 2gm powdered drug was accurately weighed in a tarred dish and dried in an oven at 105°C for one hour. It was cooled in a desiccator and again weighed. The loss on drying was calculated with reference to amount of air-dried drug and the values are recorded in **Table.3.**

Fluorescent Analysis

A small quantity of dry plant powder is placed on grease free clean microscopic slide and 1-2 drops of freshly prepared reagent solution is added, mixed by gentle tilting the slide and wait for few minutes. Then the slide is placed inside the UV chamber and observe the colour in visible light, short (254 nm) and long (365nm) ultra violet radiations. The colour observed by application of different reagents in different radiations is recorded**.** Fluorescent analysis was carried out by using the method of Chase and Pratt, (1949). Behavior was different reagents was carried out as mentioned by Johansen,(1940) and Kokoski et al.,(1958). the values are recorded in **Table. 5 & 6**

Extraction

According to the World Health Organization (WHO), nearly 20,000 medicinal plants exist in 91 countries including 12 mega biodiversity countries. The premier steps to utilize the biologically active compound from plant resources are extraction, pharmacological screening, isolation and characterization of bioactive compound, toxicological evaluation and clinical evaluation

Extraction is the crucial first step in the analysis of medicinal plants, because it is necessary to extract the desired chemical components from the plant materials for further separation and characterization. The basic operation included steps, such as pre-washing, drying of plant materials or freeze drying, grinding to obtain a homogenous sample and often improving the kinetics of analytic extraction and also increasing the contact of sample surface with the solvent system. Proper actions must be taken to assure that potential active constituents are not lost, distorted or destroyed during the preparation of the extract from plant samples. If the

plant was selected on the basis of traditional uses (Fabricant and Farnsworth, 2001), then it is needed to prepare the extract as described by the traditional healer in order to mimic as closely as possible the traditional 'herbal' drug. The selection of solvent system largely depends on the specific nature of the bioactive compound being targeted. Different solvent systems are available to extract the bioactive compound from natural products. The extraction of hydrophilic compounds uses polar solvents such as methanol, ethanol or ethyl-acetate. For extraction of more lipophilic compounds, dichloromethane or a mixture of dichloromethane/methanol in ratio of 1:1 are used. In some instances, extraction with hexane is used to remove chlorophyll (Cosa et al., 2006). As the target compounds may be non-polar to polar and thermally labile, the appropriateness of the methods of extraction must be considered. Various methods, such as sonification, heating under reflux, soxhlet extraction and others are commonly used (United States Pharmacopeia and National Formulary, 2002; Pharmacopoeia of the People's Republic of China, 2000; The Japanese Pharmacopeia, 2001) for the plant samples extraction. In addition, plant extracts are also prepared by maceration or percolation of fresh green plants or dried powdered plant material in water and/or organic solvent systems.

PREPARATION OF ETHANOLIC EXTRACT OF *Aegle marmelos* linn :(EEAM-Ethanolic extract of *Aegle marmelos* linn leaves)

PROCEDURE:

The shade dried coarsely power leaf of *Aegle marmelos* linn was defatted with petroleum ether (60 -80^{o}).the residue was dried and extracted with ethanol and water by maceration until the complete extract of the material and filtered. The extract was concentrated to obtain a solid residue (dark green).

QUALITATIVE PHYTOCHEMICAL **ANALYSIS**

Phytochemicals are chemicals derived from plants and the term is often used to describe the large number of secondary metabolic compounds found in plants. Phytochemical screening assay is a simple, quick, and inexpensive procedure that gives the researcher a quick answer to the various types of phytochemicals in a mixture and an important tool in bioactive compound analyses. A brief summary of the experimental procedures for the various phytochemical screening methods for the secondary metabolites is shown in Table. After obtaining the crude extract or active fraction from plant material, phytochemical screening can be performed with the appropriate tests as shown in the Table to get an idea regarding the type of phytochemicals existing in the extract mixture or fraction.

Preliminary phytochemical screening of the whole plant powder and aqueous , ethanol extract has been performed to detect the phytoconstituents like; alkaloid, aminoacid, carbohydrate, glycoside, mucilage, tannin, ,saponins, steroid, triterpenoid, Gums, fixed oils , fat, phenol and flavonoid were qualitatively analyzed by using the standard procedures. (Harbone J.B 1998, Nagani K et al 2012 and Kokate C.K2000).

Alkaloids

Small portion of solvent free extracts were dissolved in little dilute H_2SO_4 and filtered. The filtrate was treated with Mayers, Dragendroff, Hagers, and Wagner reagents separately. Appearance of cream, orange, brown, yellow and reddish brown precipitate in response to the above reagents respectively indicate the presence of alkaloids.

Carbohydrates

300 mg of 50% alcoholic extract were dissolved in water and filtrated. The filtrate was treated with con.H_2SO_4 and then with Molisch's regent. Appearance of pink or violet colour indicates the presence of Carbohydrates.

The filtrate was boiled with felhing's and with Benidicts solution. Formation of brick red precipitate in felhing's and benedict solution is positive result for reducing sugars and non-reducing sugars respectively.

Tannins and Phenols

Small quantity of 50% of alcoholic extract was dissolved in water and 5%ferric chloride solution or 1% gelatin solution or 10% lead acetate solution was added. Appearances of blue colour with ferric chloride or precipitation with other reagents indicate the presence of tannins and phenols.

Flavonoids

The extract mixed with few ml of alcohol was heated with magnesium turnings and then con.HCl was added under cooling. Appearance of pink colour indicates the presence of flavonoids. The extract was treated with few ml of aqueous NaOH. Appearance of yellow and change to colorless with Hcl indicate the presence of flavonoids.

Gum and Mucilage

About 10 ml of the extract was slowly added to 25 ml of absolute alcohol under constant string. Precipitation indicates the presence of gum and mucilage.

Fixed oil and Fats

A drop of concentrated extract was pressed in between two filter papers and kept undisturbed. Oil strain on the paper indicates the presence of oils and fats.

Saponins

About 1 ml of the extract was dissolved in 20 ml of water and shake in graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

Phytosterols

The extract was treated with LibermanBurchard under suitable condition. Appearance of blue-emerald green indicates the presence of phytosterols and terpenes. the result are recorded in **Table.7**

QUANTITATIVE ESTIMATION OF PHYTOCONSTITUENTS

Phenolic compounds constitute one of the main classes of secondary metabolites. They display a large range of structures and they are responsible for the major organoleptic Characteristics of plant-derived foods and beverages, particularly color and taste properties and they also contribute to then nutritional qualities of fruits and vegetables. Among these compounds, flavonoids constitute one of the most ubiquitous groups of all plant phenolic. So far, over 8,000varieties of flavonoids have been identified1.Until ~50 years ago, information on the working mechanisms of flavonoids was scare .But it has been widely known for centuries that compounds of plant origin possess abroad spectrum of biological activity(Robak Jet al.1996). In 1930,Szent-Gyorgyi isolated a new substance from oranges and

classified it as vitamin P but later, it became clear that this substance was actually a flavonoid. They can act as potent antioxidants and metal chelators. They also have long been recognized to possess anti-inflammatory, ant allergic, hepatoprotective, antithrombotic, antiviral, and anti- carcinogenic activities.

Phytochemical constituents such as tannins, flavonoids, phenols, alkaloids, coumarin and several other aromatic compounds or secondary metabolites of plants serve as a defense mechanism against predation by many microorganism, insects and herbivores. The curative properties of medicinal plants are perhaps due to the presence of these secondary metabolites Medicinal plants may be used to cure some common and other various diseases.

Estimation of total phenol content: **(Chatwal GR, 2009)**

Natural bioactive compounds like phenols and flavonoids are important secondary metabolites in plants having intrinsic properties that affect appearance, taste, odour and oxidative stability of plant based foods. These compounds also possess biological properties like antioxidant, anti-aging, anti-carcinogen, protection from cardiovascular, immune and autoimmune diseases and brain dysfunctions viz. Parkinson's, Alzheimer's, Huntington's diseases, etc.

Principle:

The total phenolic content of ethanolic extracts of *aegle marmelos linn leaves* were determined by Folin Ciocalteu reagent method. All the phenolic compounds are oxidized by the Folin-Ciocalteu Reagent and the reaction was neutralized with sodium carbonate, which is reduced during oxidation of the phenolic substances, into a mixture of blue molybdenum and tungsten oxides. The blue colour produced has a maximum absorption at about 760nm. The

absorption is proportional to the quantity of oxidized phenolic compounds. The absorbance of the resulting blue colour was measured at 760nm, using gallic acid as standard.

Instrument:

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents:

a) Folin Ciocalteu Reagent (1N) Commercially available Folin Ciocalteu Reagent (2N) was diluted with an equal Volume of distilled water. The resultant solution was kept in a brown color bottle and Stored in refrigerator at 4ºC.

b) Sodium carbonate solution (10%)

Procedure:

Gallic acid was accurately weighed and diluted in water to concentration of 1mg/mL. This solution was suitably diluted to get concentrations ranging from 2, 4, 6, 8, 10µg/ml. 0.5mL of Folin Ciocalteu reagent was added and allowed to stand for 15min. Then 1mL of 10% sodium carbonate solution was added. Finally the mixtures were mixed with distilled water and made upto10mL, allowed to stand for 30min at room temperature and total phenols were determined by spectrophotometrically at 760nm using the reagent as blank. The ethanolic extract of aegle marmelos linn leaves was weighed and diluted to get a solution of10 , 20 , 30 mcg/ml. Different concentrations of the solution were taken in separate test tubes. 0.5mL of Folin Ciocalteu reagent was added and allowed to stand for 15min. Then 1mL of 10% sodium carbonate solution was added. Finally the mixtures were mixed with distilled water and made upto10mL, allowed to stand for 30min at room temperature and total phenols were determined by spectrophotometrically at 760nm using the reagent as blank. A calibration curve was generated by plotting concentration of gallic acid versus absorbance. A linear

regression equation was determined using regression analysis. The total phenol content was calculated using the linear regression equation and expressed in terms mg of gallic acid equivalent per gm of extract (mg GAE/g). The results obtained are presented in **Table . 8 and Fig.1**

Estimation of total flavonoids content: (Mabry TJ,et al., 1970)

Principle:

The aluminum chloride colorimetric technique was used for estimation of total flavonoid estimation. Aluminum ions form stable complexes with C4 keto group and either to C3 or C5 hydroxyl groups of flavones and flavonols in acidic medium. It also forms acid labile complexes with ortho hydroxyl groups in the A or B rings of flavonoids. These complexes showed a strong absorption at 415nm which is used for the estimation of flavonoids.

Instrument:

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents:

10% aluminum chloride

1M potassium acetate

Procedure:

An aliquot quantity of rutin was dissolved in water to get a stock solution of 1mg/mL. Further dilutions were made to get concentrations ranging from 5-20 mcg/ml. 1mL of the above standard solutions were taken in different volumetric flasks, 0.1mL of aluminum chloride solution, 0.1mL of potassium acetate solution and 2.8mL of ethanol were added and

the final volume was then made up to 5mL with distilled water. After 20min the absorbance was measured at 415nm. A sample without aluminum chloride was used as a blank. From the absorbance obtained, a calibration curve was constructed by plotting concentration versus absorbance of rutin (**Fig2.)**. 1mL of ethanolic extract aegle marmelos linn at concentrations 10, 20 , 30 mcg/ml were taken and the reaction was carried out as above and the absorbance was measured at 415nm after 20min and the readings were tabulated in **Table.9** The amount of flavonoids present can be determined by linear regression analysis. The total flavonoid content was expressed as mg rutin equivalents /g of extract.

Estimation of total tannin content: **(Jain UK., et al 2004)**

Tannins are naturally occurring polyphenol compounds of varying structure. Tannins are having antioxidant and microbial activities and also used as antiseptic and astringents. They are divided into two main groups namely hydrolysable and condensed. Hydrolysable and tannins contain a polyhydric alcohol and condensed tannins are mostly flavonols.

Principle:

The tannins are estimated by Folin-Denis Method. This is based on the non-stoichiometric oxidation of the molecules containing a phenolic hydroxyl group. Tannins reduce phosphotungstomolybdic acid in alkaline solution to produce a highly colored blue solution. The intensity is directly proportional to the amount of tannins and measured in a spectrophotometer at 700nm.

Instrument:

Shimadzu UV Visible spectrophotometer, Model 1800

Reagents:

Folin-Denis reagent

10% Sodium carbonate solution

Procedure:

Prepare various concentration of ethanolic extract of *aegle marmelos linn* ranging from 10, 20, 30 mcg/ml and 0.2ml extract pipetted into test tubes. To this, 0.5ml of Folin-Denis reagent and 0.8mL of distilled water was added. The tubes were kept aside for 15min. To this, 1mL of sodium carbonate solution was added and the remaining volume was made up with 7.5mL of distilled water. Then the tubes were shaken and the absorbance was recorded at 700nm after 30min. Tannic acid, used as a standard was taken at different concentration 2, 4, 8, 12, 16, 20mcg/ml in different test tubes and the procedure adopted above was followed. The calibration curve for tannic acid was plotted using concentration versus absorbance (**Fig.3).** A linear regression equation was calculated and the equation was used to calculate the amount of total tannins as tannic acid equivalent. The amount of tannin content is expressed in mg/g of extract. The results obtained are presented in **Table.10**

Estimation of total coumarin content :(Osorio 0.k et al., 2004)

Principle:

The borntrager reaction is based on the solubility of free coumarin derivatives in polar organic solvents and the solubility of their soluble alkali phenotes. Coumarin absorbs at 280 nm, ionisations of phenolic hydroxyls in the molecules by alkaline hydroxide causes a bathochromic deviation to 320 nm, which is proportional to the coumarin concentrations.

Reagents:

1.1,2 benzopyrone (1 mg/ml)

2. Lead acetate (5%, w/v)

3. Hydrochloric acid solutions (0.1M)

4. Methanol (80%)

Instrument:

Shimadzu UV Visible spectrophotometer, Model 1800

Procedure:

The calibration curve must be prepared using 50-500 microlitres of aliquots of the coumarin solutions with 2 ml of distilled water and 500 micro litre of lead acetate solution. The sample should be shaken and the final volume adjusted to 10 ml with distilled water .take 2 ml solution add 8 ml hydrochloric acid solution and absorbs the range at 320 nm. To quantify the coumarin 500 microlitre of the extract should be transferred to the test tube , next 2 ml of distilled water and 500 micro litre of lead acetate solution will be added .the sample is shaken and then 7 ml of distilled water are added before transferring 2 ml of this solutions to a new test tube and adding 8 ml of hydrochloric solutions . the sample remain at room temperature for 30 mins , the spectrometer should be adjusted to a 320 nm wavelength and the equipment must be performed in at least triplicate and the total coumarin equivalents per gram of the sample extract(mg ce/g). the results are recorded in **Table.11 and fig 4.**

CHROMATOGRAPHY

Chromatography is a non-destructive procedure for resolving a complex mixture into its individual fractions or compounds. "Chromato" "graphy" derives its name from two words as chromo= colour and graphy= writing. I.e. colour bands are formed in the procedure which

are measured or analyzed. It is defined as the process of separation of the individual components of a mixture based on their relative affinities towards stationary and mobile phases. These two phases can be solid-liquid, liquid-liquid or gas-liquid.

Principle

The samples are subjected to flow by mobile liquid onto or through the stable stationary phase. The sample components are separated into fractions based on their relative affinity towards the two phases during their travel. The fraction with greater affinity to stationary layer travels slower and shorter distance while that with less affinity travels faster and longer. Overall available **chromatography techniques** for regular analysis include,

a) Column chromatography.

b) High performance liquid chromatography.

c) Gas chromatography.

d) Ion-exchange chromatography.

e) Size exclusion chromatography.

f) Thin layer chromatography.

g) High performance thin layer liquid chromatography.

h) Paper chromatography.

THIN LAYER CHROMATOGRAPHY :()

The term “thin-layer chromatography”, introduced by E. Stahl in 1956, means a chromatographic separation process in which the stationary phase consists of a thin layer applied to a solid substrate or “support”. Thin layer chromatography (TLC) and high performance thin layer chromatography (HPTLC) – now also called planar chromatography.

Thin-layer chromatography or TLC, is a solid-liquid form of chromatography where the stationary phase is normally a polar absorbent and the mobile phase can be a single solvent or combination of solvents. TLC is a quick, inexpensive microscale technique that can be used to:

• determine the number of components in a mixture

• verify a substance's identity

• monitor the progress of a reaction

• determine appropriate conditions for column chromatography.

• analyze the fractions obtained from column chromatography.

Principle

TLC is based on the principle of adsorption. The separation depends on the relative affinity of compounds towards stationary and mobile phase. The compounds under the influence of mobile phase (driven by capillary action) travel over the surface of stationary phase and during this movement, the compounds with higher affinity to stationary phase travel slowly while the others travel faster. Thus separation of components in the mixture is achieved.

Preparation of TLC Plates

The adsorbent (silica gel G) slurry was prepared in water in the ratio of (1: 2). The glass plates (20cm x 5cm) were cleaned and laid in a row as a template, the suspension was poured into Stahl TLC spreader, which was adjusted to 0.25mm thickness and coated in a single passage of the spreader over them. These plates were air dried and activated in hot air oven at 105ºC for 30min and kept in a dessicator. The plates were used as the stationary phase

or Pre-coated aluminum plates coated with silica gel G F_{254} (Merck) were also used for analysis.

Sample application

The extracts were dissolved in mobile phase and the spot was applied on the TLC Plates using capillary tube.

Development of the chromatogram

After drying of the spot, the plates were developed in a chromatographic tank containing the solvent system. After one third of the plate was developed the plates were taken outside and dried. The TLC plate was examined visually or under UV light.

Solvent system I

Stationary phase - Silica gel G

Mobile phase - Toluene : Ethyl Acetate : Formic acid (8:2:0.0.01)

Detecting agent - visual & UV light

Solvent system II

Stationary phase - Silica gel G

Mobile phase – Benzene:Acetone (9:1)

Detecting agent - visual & UV light

The R_f values were calculated using the formula [Distance travelled by solute/ Distance travelled by solvent]. The phytochemical evaluation of ethanolic extract of *Aegle marmelos* linn leaves was carried out by using TLC studies. The results are presented in **Table.12** and **Plate .10 &11**

ISOLATION AND CHARACTERIZATION OF PHYTOCONSTITUENTS:

The chemical investigation of a plant involved Collection and proper identification of the plant materials, extraction, fractionation, purification &isolation of compounds and structural characterization of the purified compounds. Various chromatographic techniques were utilized for isolation and purification of the plant constituents. On the other hand, the structures of the purified compounds were determined by extensive analyses of UV, IR, and NMR. (Mohamed, . et al.)

Many aqueous extract or alcoholic extracts, hydro alcoholic extracts are used in manufacturing Ayurvedic and herbal formulations. If the phytochemical profile of the plant or its part is known an appropriate kind of extract can always be used by selection for a particular purpose. A TLC or HPTLC profile of the phytochemical can be employed for the similarity or dissimilarity or to find out the presence or absence of the certain phytochemicals (Indian herbal pharmacopoeia). TLC/HPTLC has excellent resolution and, therefore, permits simultaneous identification of a wide range of substances in a single run.(Rahul Kasar., et al 2013.)

Column chromatography is an isolation technique in which the phytoconstituents are being eluted by adsorption. The principle involved in this separation of constituents is adsorption at the interface between solid and liquid. The component must have various degree of affinity towards adsorbent and also reversible interaction to achieve successful separation. No two compounds are alike in the above aspect. Low affinity compounds will elute first. The columns of different sizes were used for the present studies. Since the ethanolic extract was found to possess significant pharmacological activity when compared to other extracts an attempt was made to fractionate the ethanolic extract by column chromatography. The elution

was done by using solvents of different polarity like n- hexane, ethyl acetate, carbon tetrachloride, methanol and water

Materials and Methods

Apparatus required:

Silica gel 60 -120 Mesh

Column Chromatography glass apparatus.

Measuring cylinders (100ml, 500ml capacity)

Beakers,

Distillation apparatus,

Screw caped container.

Chemicals required:

Pet.ether

Chloroform

ethanol.

Procedure

The Ethanolic leaves extract aegle marmelos linn was of subjected to Silica gel column chromatography for the isolation of the phytoconstituents. An appropriate column sized 5cm diameter and 50cm length was used. It was washed with water and rinsed with acetone and then dried completely. Little of pure cotton was placed at the bottom of column with the help of a big glass rod. Solvent hexane was poured into the column up to ¾thuntil it became free flowing powder. When it reached a defined state it was slowly poured into the

column containing hexane solvent with slight movement of stirring by glass rod to avoid clogging. Little cotton was placed on top of silica gel- extract mixture pack to get neat column pack. The knob at the bottom was slowly opened to release the solvent. The elution was done using pet.ether, chloroform, ethanol. In the ratio of pet.ether: chloroform (2:1), chloroform: ethanol (9:1,4:1,2:1).All the broad fractions were collected separately and subjected to TLC. The solvents were evaporated by rotary vacuum evaporator.(

Isolation of Compound

The three fraction chloroform: ethanol on concentrating and evaporator yielded greenish yellow was obtained. It was then recrystallized with methanol filtered and the yellowish white powder was dried. The pure compounds obtained were then subjected to spectral analysis for the determination of the structure of the compound. The result are tabulated in table.13 &14

Thin layer chromatography:

As soon as the fractions were eluted, it was analyzed by using preparative TLC plate with suitable mobile solvent The developed chromatogram was observed under UV and also derivatized with detecting agent.The result are tabulated in table.15, And the photograph presented in plate.12

Chemical test:

Test 1: few ml of fraction was evaporated to dryness in a vessel and the residue was dissolved in hot distilled water. It was then cooled and divided into two test portions, one was reference, second was the test. To the second test tube added 0.5 ml of 10% ammonium hydroxide. Occurrence of intense fluorescence indicates the presence of coumarins and derivatives (Jagessar RC et al.,2010)

Test 2: To the concentrated fraction, added few drop of alcohol FeCl3 solution deep green colour formed which turned to yellow on addition of conc. HNO3 indicates the presence of coumarins.**(**Govindappa M, et al ., 2015**)**

Test 3: The fraction was mixed with 1 N NaOH solution (one ml each). Development of blue green fluorescence indicates presence of coumarins. **(**Govindappa M **,** et al ., 2015**)**

The chemical test result are tabulated in table.16

SPECTRAL ANALYSIS:

The isolated compounds were taken to determine the structure by

Instrumental and spectral analysis such as

UV spectroscopy

IR spectroscopy

UV Spectroscopy

UV spectroscopies have been in general use for the last 35 years and over this period have become the most important analytical instrument in modern day laboratories. It is one of the most frequent methods for plant drug analysis. This method involves the measurement of the amount of UV radiation (190-380 nm) absorbed by a substance in solution.

Ultraviolet and visible absorption spectra offer a useful source of supporting evidence in the elucidation of structures of organic compounds. Moreover, selective absorption spectrum serves as an identifying fingerprint for a particular structure in many cases.

The value of the UV and Visible spectra in identifying unknown isolated compounds is indicative of the compounds .The max and absorbance of the isolated compound from the chloroform: ethanol fraction of the ethanolic leaves extracts of *Aegle marmelo* linn were

recorded using shimadzu 1800 UV visible spectrometer. The result are tabulated in **table.17** and spectrum show in **fig.5**

3.2.6. IR Spectroscopy

FTIR has proven to be a valuable tool for the characterization and identification of compounds or functional groups (chemical bonds) present in an unknown mixture of plants extract (Eberhardt et al., 2007; Hazra et al., 2007**)**.Mechanism of bond stretching and bending is happened when electromagnetic radiation ranging from 500cm−1 to 4000 cm −1 passed through sample. Instrument used was FTIR shimadzu spectrometer.

In addition, FTIR spectra of pure compounds are usually so unique that they are like a molecular "fingerprint". For most common plant compounds, the spectrum of an unknown compound can be identified by comparison to a library of known compounds. Samples for FTIR can be prepared in a number of ways. For liquid samples, the easiest is to place one drop of sample between two plates of sodium chloride. The drop forms a thin film between the plates. Solid samples can be milled with potassium bromide (KBr) to and then compressed into a thin pellet which can be analyzed. Otherwise, solid samples can be dissolved in a solvent such as methylene chloride, and the solution then placed onto a single salt plate. The solvent is then evaporated off, leaving a thin film of the original material on the plate. .the result are tabulated in **table.18** and spectrum show in **fig.6**

PHARMACOLOGICAL STUDIES:

1. In vitro antioxidant activity methods

1. a) Reducing power assay :(Jayanthi P and Lalitha P 2011)

Principle:

This is a spectrophotometric method and is based on the principle that an increase in absorbance of the reaction mixture as concentration increase indicates an increased antioxidant activity. The assay is based on the reduction of ferric in potassium ferricyanide to ferrous to form potassium Ferrocyanide by the sample and the formation of Prussian blue colour complex when treated with ferric chloride. The absorbance of the blue complex is measured at 700nm.

Instrument:

Shimadzu UV Visible spectrophotometer. Model 1800

Reagents:

1% potassium ferricyanide

10% trichloro acetic acid.

0.2M, phosphate buffer (pH 6.6)

0.1% ferric chloride

.

Procedure:

Various concentration of ethanolic extract of *aegle marmelos linn* was mixed with 0.75mL phosphate buffer and 0.75mL potassium ferricyanide [K_3 Fe(CN_6)], then the mixture was incubated at 50°C for 20 min. 0.75mL of trichloro acetic acid was added to the mixture, which was then centrifuged at 3000rpm for 10min. Finally 1.5mL of the supernatant solution was mixed with 1.5mL of distilled water and 0.1mL of ferric chloride ($FeCl_3$) and absorbance was measured at 700nm in a UV-Visible Spectrophotometer. Ascorbic acid was used as standard and phosphate buffer used as blank. The absorbance of the final reaction mixture of three parallel experiments was expressed as mean ± standard error of mean. Increased absorbance of the reaction mixture indicates stronger reducing power. The results obtained are tabulated in **table. 19 .**and the graphical representation is presented in **Fig.7&8**

1. b) Total antioxidant activity by Phosphomolybdenum Method:

(Laloo D and Sahu AN., 2011)

Principle

Total antioxidant capacity was measured by spectrophotometric method of Prieto *et al*. Phosphomolybdenum method assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of green phosphate Mo (V) complex at acidic pH and the absorbance was measured at 695nm. This method is used to determine the total antioxidant activity of samples.

Reagents

0.6M sulphuric acid

28mM sodium phosphate

4mM ammonium molybdate

Instruments

Shimadzu UV Visible spectrophotometer, Model 1800

Procedure:

An aliquot of 0.3mL of different concentrations of sample solution was combined with 2.7mL of the reagent solution (H2SO4, sodium phosphate and ammonium molybdate). In case of blank, 0.3mL of methanol was used in place of sample. The tubes were incubated for 95°C for 90min. After the mixture was cooled to room temperature, the absorbance was measured at 695nm against blank. Ascorbic acid was used as a standard and was treated in a similar manner. The total antioxidant activity is expressed as the number of equivalents of ascorbic acid (μg/g). The results were tabulated in **table.20 and** the graphical representation is presented in **Fig.9**

INVITRO& INVIVO PHOTOPROTECTIVE ACTIVITY

The harmful effects of solar radiation are caused predominantly by the ultraviolet (UV) region of the electromagnetic spectrum, every year, about one million people are diagnosed with skin cancer and about 10.000 die from malignant melanoma. Most skin cancer occurs on the areas of the body that are most frequently exposed to the sun, such as the face, neck, head and back of the hands (SAX, 2000). Due to these facts, sunscreens substances are now incorporated into everyday products such as moisturizers, creams, lotions, shampoos, mousses, and other hair and skin preparations. The regular use of these products may help to reduce the chance of the harmful effects of ultraviolet radiation. However, it is necessary that a very efficient sunscreen substance is used in the cosmetic formulation. The efficacy of a sunscreen is usually expressed by the sun protection factor (SPF), which is defined as the UV energy required producing a minimal erythema dose (MED) on protected skin, divided by the UV energy required to produce a MED on unprotected skin.

The minimal erythemal dose (MED) is defined as the lowest time interval or dosage of UV light irradiation sufficient to produce a minimal, perceptible erythema on unprotected skin (Wood *et al*., 2000; Wolf *et al*., 2001). The higher the SPF, the more effective is the product in preventing sunburn. Nevertheless, it is necessary to standardize methods to determine the SPF of these products.

2. IN VITRO: SUNPROTECTION FACTOR DETERMINATION BY ULTRA VIOLET SPECTROSCOPY :

Reagents:

Ethanol, analytical grade

Water

Apparatus:

Beckman UV/Visible spectrophotometer,

Equipped with 1 cm quartz cell,

.

Methods:

Sample preparation:

- 100 mg of all ethanolic extract are weighed, transferred to a 100 mL volumetric flask, diluted to volume 100 ml with ethanol, followed by filtered through cotton, rejecting the ten first ml. from the above solution take 10 ml and make up to 100 ml with ethanol .from above stock solution make (2 mcg, 4 mcg, 6 mcg) concentration .The absorption spectra of extract solution were obtained in the range of 290 to 320 nm using 1 cm quartz cell, and ethanol as a blank. The absorption data were obtained in the range of 290 to 320, every 5 nm, and 3 determinations were made at each point, followed by the application of Mansur equation. Mansur *et al.* (1986), developed a very simple mathematical equation which substitutes the *in vitro* method proposed by Sayre *et al.*, (1979), utilizing UV spectrophotometry and the following equation. (Manoj A, Suva,2014).the result are tabulated in **table.21,22,23**.,24 and spectrum show in **fig.10.**

$$\mathbf{SPF= CF\times\sum_{290}^{320} EE \times I \times Abs}$$

Where: EE (l) – erythemal effect spectrum; I – solar intensity spectrum; Abs - absorbance of sample, CF – correction factor (= 10).

WAVELENTH(nm)	EE*I (normalized)
290	0.0150
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0839
32o	0.0180

INVIVO UVA PHOTOPROTECTIVE ON EEAM LINN LEAVES USING ZEBRA FISH EMBRYO ASSAY:

Zebrafish make excellent research models:

1. Genetic similarity to humans

Zebrafish are vertebrates and hence share a high degree of structure and functional homology with mammals, with humans. Due to the maintenance of cell biological and developmental processes across all vertebrates, studies in fish can give great understanding into human disease processes. For example, to date all proteins studied have a comparable role in fish and mammals.

2. Easier to house and care for than rodents

Due to their small size and the comparatively simple nature of their natural environment, it is easier to retain zebrafish in what appear to be more natural conditions than it is possible to simulate for mammals. This minimizes housing stress and the influence such stress may have on the outcome of experiments. Not only does this add to the refinement of animal usage, it also minimizes the number of animals that need to be used because it reduces the between subject variation that can be caused by stress.

3.Impact of any genetic mutation or drug treatment is easy to see

Zebrafish embryos and larvae are entirely transparent, meaning that it is possible to follow the impact of a genetic manipulation or pharmacological treatment using noninvasive imaging techniques. Less disturbing techniques minimize animal suffering. The invasive procedures avoided not only impact on welfare, but may also affect the experimental outcome, so the transparent nature of zebrafish larvae could also mean results are more accurate and easier to reproduce.

4. Lots of offspring

Ensuring a ready supply of animals for research is also easier with zebrafish. Zebrafish have a much larger number of offspring in each generation than rodents. Rodents have 5- 10 offspring per pairing, in comparison to the 200-300 obtained from fish. Zebrafish offspring also grow and develop very quickly.

5. Easier to introduce genetic changes

Zebrafish embryos are able to absorb chemicals that have been added to their water, meaning it is easy to introduce changes to their genes using nothing more than chemical mutagens. Zebrafish are able to tolerate much higher levels of chemical mutagens than can be

tolerated by rodents so it is possible to induce a much higher density of mutations in their genome.

PRIMARY TOXICOLOGICAL STUDY:

INTRODUCTION:

Zebra fish,(*Danio rerio)* are vertebrate organisms that are an excellent alternative model development, permeability to small molecules, genetic similarity to humans, and great fecundity. Zebrafish embryos develop most of the major organ systems present in mammals, including the cardiovascular, nervous and digestive systems in less than a week.(Westerfield 1995) Zebrafish are also an excellent biomedical model because 70% of human genes have at least one obvious zebrafish orthologu. This has also become an attractive model because of the possibility of performing small-scale, high-throughput analyses(de castro 2009).

3. a)EMBRYO TOXICITY STUDY :(Mindy Reynolds 2013)

MATERIAL:

Fertile egg, E_3 Embryo medium, dimethyl sulphoxide, glasss petridishes, research microscope, incubator, micropipette, ethanolic extract of *Aegle marmelos,* standared caffeine.

Method:

The eggs were transferred to each of the glass petri dishes (2-3 per dishes) containing different concentration of EEAM (0.5 , 0.75 , 1 , 2 mcg/ml) dissolved in 0.1% of DMSO and DMSO act as control . Embryo medium served as the overall control. Standard caffeine of

concentration 10 mcg/ml was taken as positive control. The maximal acceptable toxicant concentration (MATC) was calculated by scoring the malformations.(Dave , G et al 1987).

The development of blastula eggs was monitored at specified time points (12, 24, 36, 48hrs,) under microscope. End points were used for assessing the effect of drug during the major organ is visible included edema, eye malformation, bent tail and death. Malformations were also noted and described among the 0.1% DMSO treated and standard caffeine. the values are recorded in **Table. 25 ,26,27**

3. b) INVIVO UVA PHOTOPROTECTIVE ON EEAM LINN LEAVES USING ZEBRA FISH EMBRYO ASSAY:

(Maria Guinea et al., 2012)

Embryo harvesting and maintenance:

.Extract treatment:

One hour before irradiation, 2 to 4 hpf embryos were placed in 96-well plates (Deltalab, Spain) containing 50µl of either egg water or egg water supplemented with different concentration of *Aegle marmelos* extract (AM) was were dissolved in egg water when required the final pH was adjusted to 7.0. Egg water containing 0.1% DMSO was also used as a vehicle control in all experiments as well as a spawn control. In parallel to the photoprotection assay, toxicity was assessed using plates with embryos incubated with extracts at the same concentration but without exposing to UV irradiation.

. Radiation exposure

After 1 h of incubation at 28 _C, embryos were irradiated for 6 min with a 365- nm UV lamp. After irradiation, the medium was removed and 50 micro litre of fresh egg water was added to wells containing the embryos. Plates containing UVA -radiated embryos or non-irradiated were incubated at 28^{o} C for up to 48 hpf to evaluate morphological abnormalities and survival.

Morphological analysis:

Embryo morphological features were observed at 48 hpf using a research microscope () at 40x magnification. UV-induced morphological defects included: swollen yolk, edema, abnormal pigmentation, kinked down tip tail and twisted tail. Due to the seriously affected fin development (Dong et al., 2007).. A characteristic example of each category is shown in **plate.13-17** and the values are recorded in **Table. 28, 29, 30,31**

CHAPTER-5

RESULT AND DISCUSSION

CHAPTER-5

RESULTS AND DISCUSSION:

Pharmacognostical evaluation:

Pharmacognostical works are ensuring the quality and purity of starting material by evolving standards for comparison. To ensure reproducible quality of medicinal plants, authentication is essential. Pharmacognostical parameters like Leaf constants; microscopy and physiochemical parameters are the basic standardizations of medicinal herbs. According to WHO, the Macroscopial and Microscopical description of medicinal herbs is the first step towards the confirmation of identity and purity of such material. The pharmacognostical parameters are major reliable and inexpensive criteria for confirmation of crude drugs.

MACROSCOPICAL EVALUATION:

Morphological studies on the leaves of *Aegle marmelos* linn:

The Macroscopial examination revealed that leaves are green in color, attenuate, trifoliate very rarely five foliate. The shape of the leaves is lenceolate. The size is varying ranging from 3 cm to 5 cm in length and 2 to 4 cm in width. The margin is crenate with acuminate apex and symmetric base with petiole around 2.3 to 6.5 cm long. .leaf lets subsessile and it terminal long petiole Surface of leaves is glabrous, Surface of leaves pellucid-punctate and shiny showing parallel venation with brittle texture. Leaves bear aromatic Odour and characteristic taste. The Macroscopial character result are tabulated in **Table no: 1 and plate no 1 to 3**

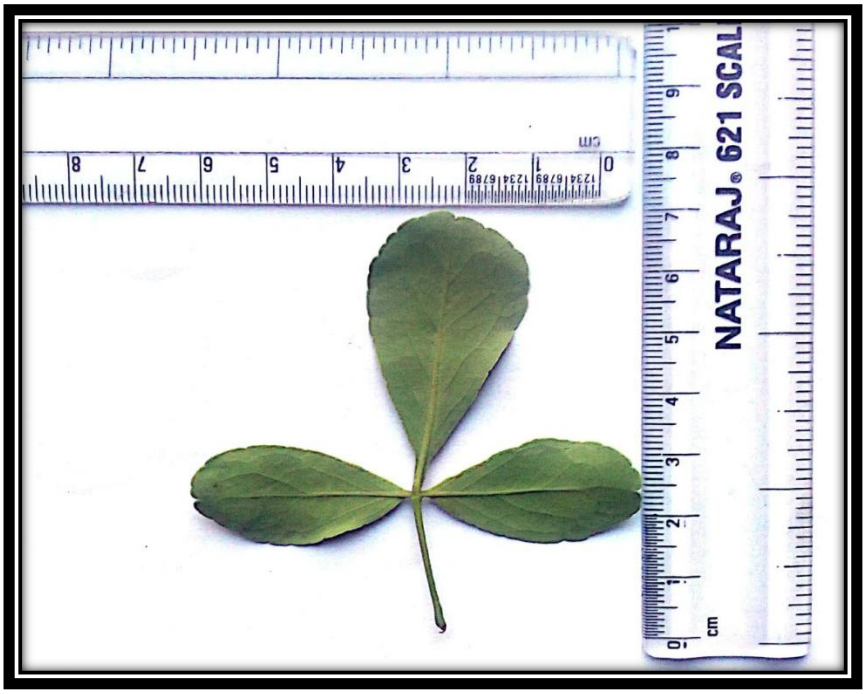
PLATE NO:1

Habitat

PLATE NO: 2

DORSAL VIEW OF LEAVES OF AEGLE MARMELOS LINN:

PLATE NO: 3



VENTRAL VIEW OF LEAVES OF AEGLE MARMELOS LINN:

Table: 1 Macroscopial studies of *Aegle marmelos* linn leaves

S.no	Parameters	Observations
1	Colour	Pale (or) dark green
2	Odour	Characteristic
3	Taste	Bitter
4	Apex	Acuminate
5	Margin	Crenate
6	Arrangement	Alternate
7	Shape	Trifoliate
8	Leaf length	3-4.5 cm
9	Leaf width	2-2.5 cm
10	Petiole Length	2.5-6.5 Cm
11.	Base	Symmetrical

The cytomorphological studies broadcast the diagnostic characters of *Aegle marmelos* linn leaves are trifoliate leaves; pellucid-punctate surface and showing parallel venation with brittle texture; lateral subsessile and it terminal long petiole. These morphological tools are supportive for identification, authentication, and distiquish from the relevant species of the genus.

MICROSCOPICAL EVALUATION:

Microscopical studies on leaves of *Aegle marmelos linn:*

The slides of T.S of leaf of plant were prepared and subjected to microscopical examination. The histology of different parts of plant was examined and the observations were recorded.

The leaves of *Aegle marmelos* linn was subjected to Microscopial and result are presented in **plate no: 4 to 8**

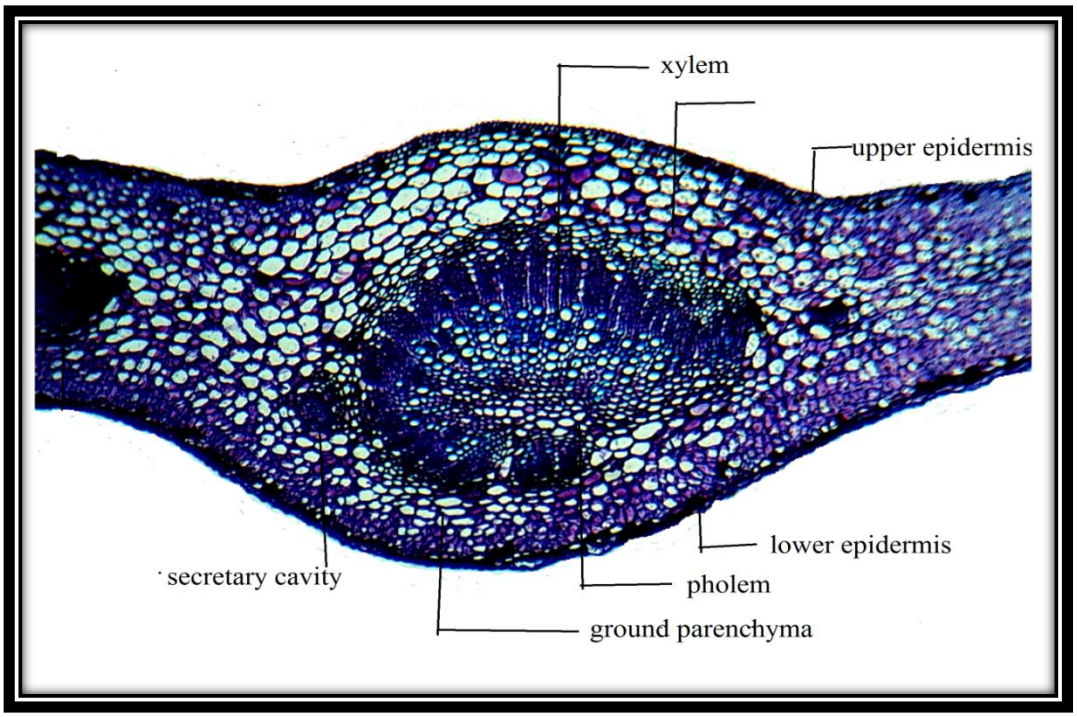
Leaf:

Leaf is a narrow and thick midrib and wing like lamina **(Plate.4.)**. Midrib portion shows continuation of the epidermal layer of lamina over midrib. Below the upper epidermis and above the lower epidermis are seen strips of the collenchyma (3-4 layered). The vascular bundle of the midrib is large, somewhat circular and collateral. Midrib shown the presence of xylem and phloem arranged in an arc.

Lamina: (Plate. 5)

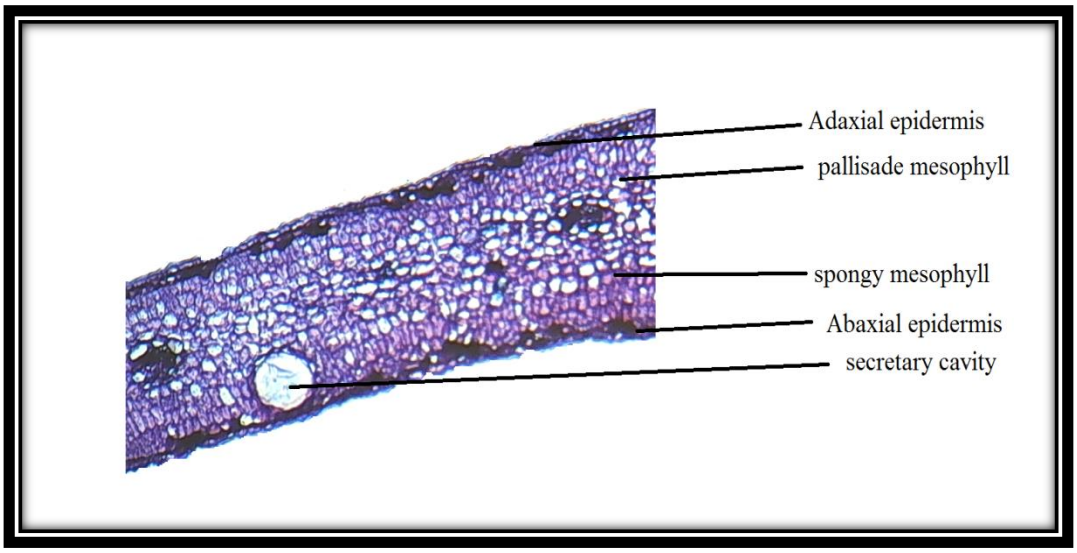
The lamina portion of the T.S of *A. marmelos* leaf showed the presence of upper and lower epidermis both comprised of round to oval shaped cells. The Epidermis is single layered occasionally interrupted with sunken stomata on both surfaces and over lined by a thick layer of cuticle. Both upper and lower epidermal layers bear stomata. Each stoma has two guard cells and two subsidiary cells and they correspond to paracytic type. Numbers of stomata are high in upper epidermis compared to lower epidermis and both epidermis shown presence of covering trichrome. Interior to the epidermis is a many layered palisade tissue, which consists of closely, packed oval cell without much intercellular space.

PLATE NO: 4



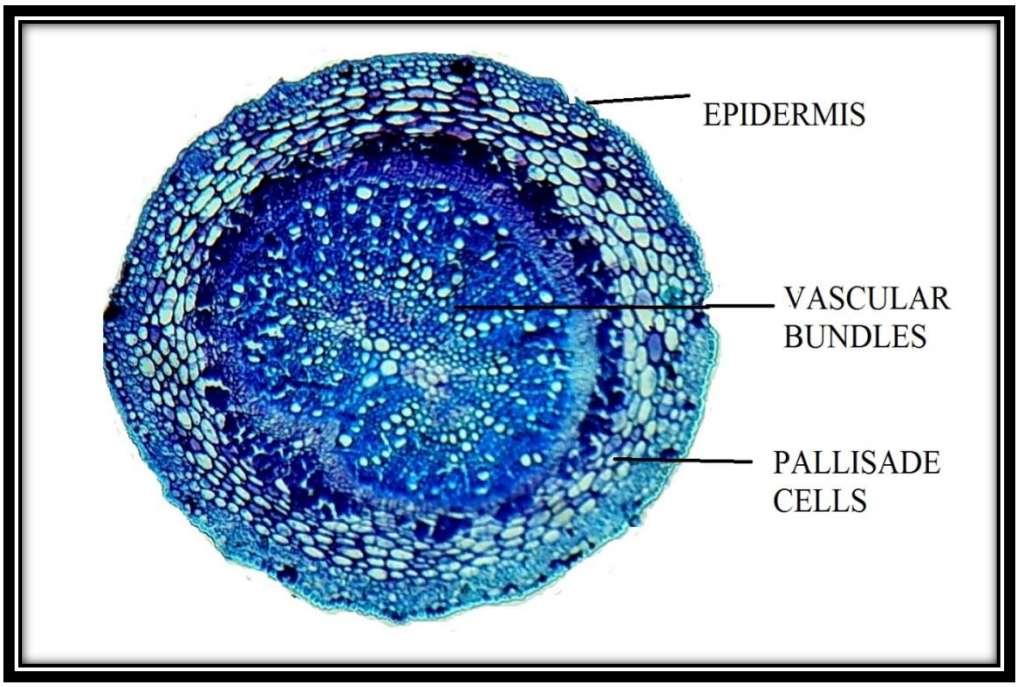
T.S OF MIDRIP OF LEAVES AEGLE MARMELOS LINN:

PLATE NO: 5



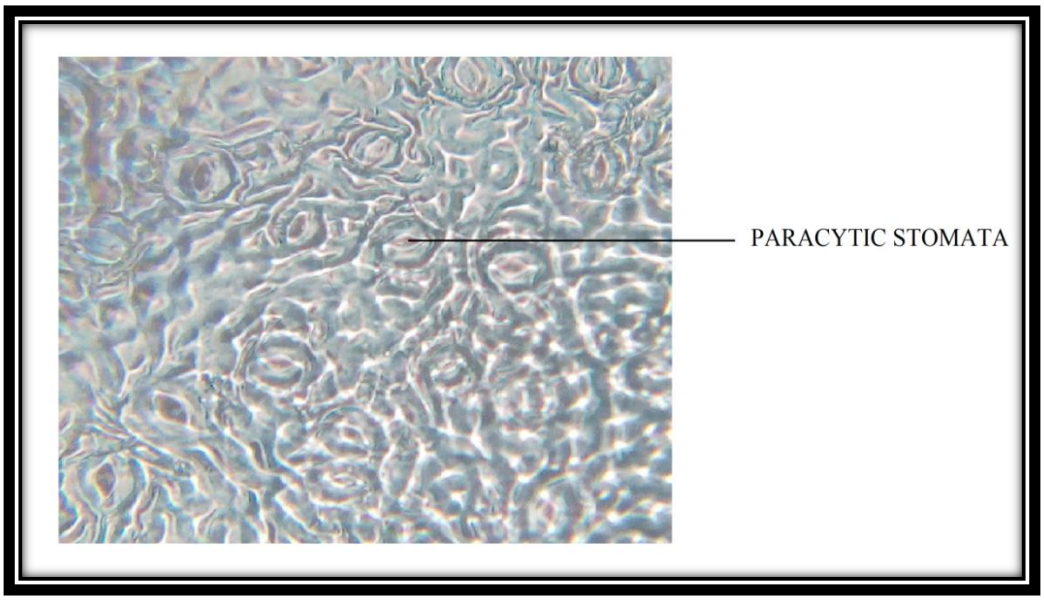
T.S OF LAMINA OF AEGLE MARMELOS LINN

PLATE NO: 6



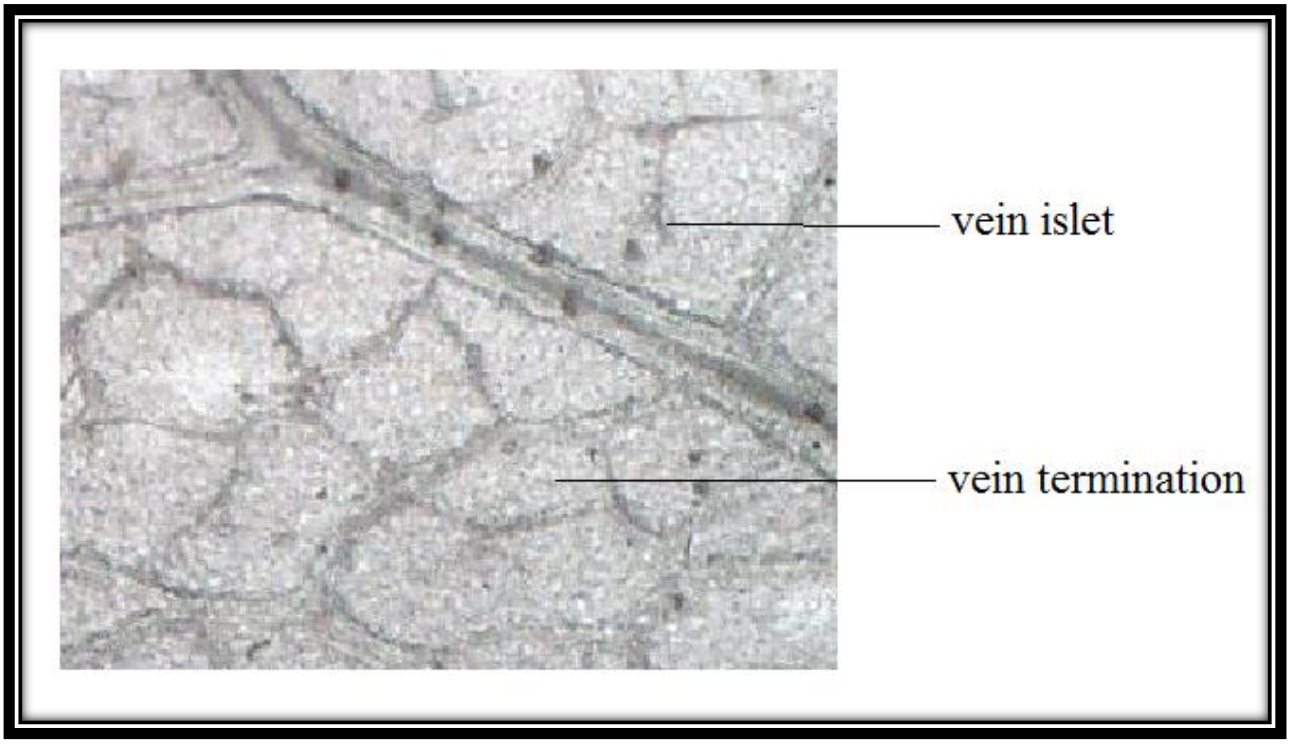
T.S OF PETTIOLE OF AEGLE MARMELOS LINN LEAVES

PLATE NO: 7



STOMATA

PLATE NO:8



VENATION PATTERN OF *AEGLE MARMELOS* LIN N LEAVES

Petiole: (plate: 6)

In T.S., Petiole is broad 'C' shaped in outline with a single layer of schizogenous cavity and a conspicuous broad. 'C' shaped vascular bundle in the centre. Epidermis is single layered occasionally interrupted with sunken stomata on both surfaces and over- lined by a thick layer of cuticle. Interior to the epidermis is a many layered palisade tissue, which consists of closely, packed oval cell without much intercellular space.

Epidermal cells and Stomata: (Plate.7)

The epidermal cells are small with highly wavy anticlinal walls. The stomata are abundant; they are oriented in one plane. The stomata are paracytic type with two subsidiary cells lying on either side of the stomata and being parallel to the guard cells. The stomata are elliptical with prominent stomata pores the guard cells

Venation of the Lamina: (Plate.8)

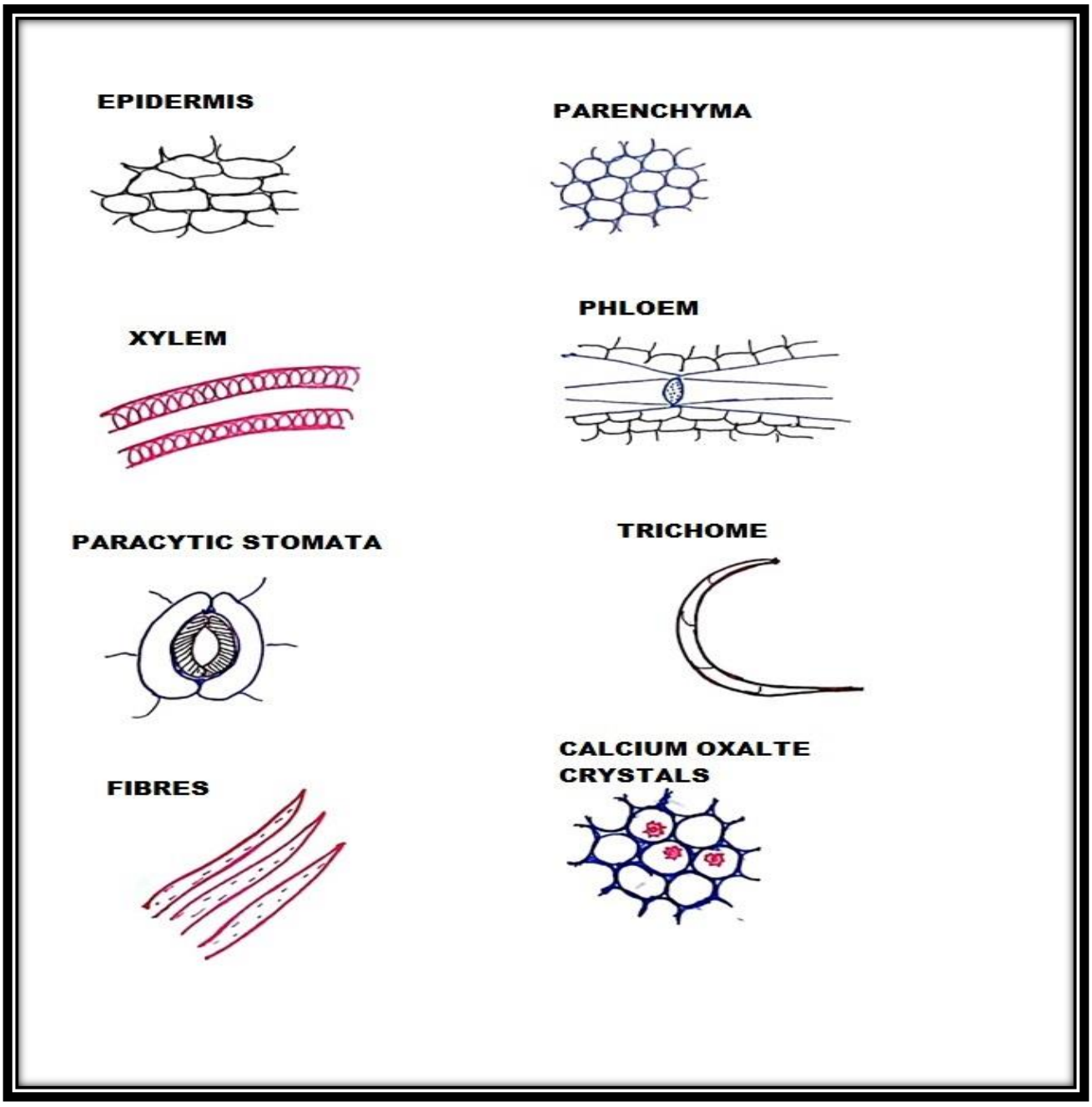
The leaf consist of thin, less prominent lateral veins forming dense reticulations .The vein- islets are narrow, rectangular in outline and the vein boundaries are fairly distinct. Vein terminations are present in most of the islets. The vein terminations may be simple (unbranched) or branched once. Calcium oxalate crystals of raphides are fairly common in the mesophyll tissue.

POWDER MICROSCOPY:

The various characteristic features observed when seen under a microscope are Presented in **Plate n**o**.9** The features observed under a microscope include stomata, thick walled covering trichrome, xylem and phloem vessels.

The powder microscopy of leaves of *A. marmelos* leaves showed groups of fibers with calcium oxalate crystals and also exhibit outer and inner epidermis with round to oval cells, covered with striated cuticle. Composed of xylem and phloem arranged in an arc. The leaves show paracytic stomata, Calcium oxalate crystals were numerous and mainly of cluster crystal type. It contains numerous covering trichrome scattered in the powder. Some xylem vessels (pitted vessels) were also visible which were lignified. Cells of palisade and spongy parenchyma were also visible.

PLATE NO: 9:



POWDER MICROSCOPY OF AEGLE MARMELOS LINN LEAVES:

Quantitative Microscopy of leaves of *Aegle marmelos* linn:

The parameters such as vein termination, vein islet and stomatal numbers, stomatal index and palisade ratio of the leaf of *A. marmelos* were observed and recorded. The observations and results are summarized in the Table.2

Table: 2 **Quantitative microscopy of *Aegle marmelos* linn leaves**

s.no	Parameters	Minimum (Per $mm^{2)}$	Average (Per$mm^{2)}$	Maximum (Per$mm^{2)}$
1	Vein islet number	9	11.2	14
2	Vein termination number	7	8.4	10
3	Stomatal number			
	Upper epidermis	6	9.6	12
	Lower epidermis	5	6	9
4	Stomatal index			
	Upper epidermis	12	15.4	16
	Lower epidermis	8	10	12

From the table, it can be observed that the number of stomata in the upper epidermis was found to be 9.6 and the lower epidermis was found to be 6. The stomatal index observed in epidermis was found to be 15.4 and the stomatal index in lower epidermis was found to be10. The vein islet number was found to be 11.2 and the vein termination number was8.4. the stomatal number and stomatal index in upper epidermis high compared to lower epidermis. The values help in identification of leaf of *Aegle marmelos linn* from the genus *Aegle* since these values are unique for each plant.

Physiochemical Evaluation of leaves of *Aegle marmelos linn*:

Physiochemical parameters are helpful in finding the purity and quality of the powdered drug. The powdered drugs were evaluated for its physiochemical parameters such as loss on drying, ash value and extractive values. The results were compiled in **Table -3**

Table: 3 **Determination of physio- chemical constants of *Aegle marmelos* linn leaves:**

s.no	Physio – chemical constant	Reports W/W %
1	Foreign organic matter	0.0295%
2	Loss on drying	0.67%
3	Petroleum ether extractive value	5.6%
4	Chloroform extractive value	4.4%
5	Methanol extractive value	9.2%
5	Ethanol extractive value	7.6%
6	Aqueous extractive value	5.2%
7	Hydro -alcoholic extractive value	8.6%
8	Total ash	6.33%
9	Water soluble ash	1.18%
10	Acid soluble ash	2.09%

It can be seen that the foreign organic matter present in the crude material was very low. The percentage of total ash was found to be 6.33 and the percentage of water soluble ash was found to be 2.09 while the acid insoluble ash was 1.18 ±0.074. The determination of ash values helps to find out where the powdered material was adulterated with sand and other inorganic material. The water soluble ash helps us to find the amount of inorganic material present in the crude drug, while acid insoluble ash helps us to find the amount of sand and other debris in the crude material. The various extractive values with different solvents have

been determined. A maximum extractive value was found with methanol (9.2 %) followed by hydro alcohol (8.6 %). The extractive value helps us to decide what solvent will be useful for extraction of maximum active principle and also helps to decide whether the crude material has already been exhausted or not.

Organoleptic evaluation

The organoleptic evaluation of leaves *Aegle marmelos* linn powder are tabulated in **Table no.4**

Table.4: Organoleptic character of *Aegle marmelos* linn leaves powder

S.no	Name of the Character	Observation
1.	Nature	Coarse
2.	Colour	Dark Green
3.	Odour	Aromatic
4.	Taste	Bitter

Organoleptic characters such as colour, nature, taste and odour of the plant were used as identification features. The Colour of the *Aegle marmelos* linn leaves are dark green , coarse in nature , characteristic odour , bitter in taste. Thus the organoleptic characters of the plant powder could be used as diagnostic character.

FLOURESCENTS ANALYSIS:

The powdered crude drug analysis was aimed to study and also to assess the quality of herbal drugs for therapeutic value which are generally studied by classical pharmacognostical Studies. The authenticity of herbal drug was confirmed by comparison of their powder characters. The organic molecules absorb light usually over a specific range of wavelength

and many of them remit such radiation. So if the powder was treated with different reagents and seen in the uv chamber, different colour will be produced.

The powder taken organoleptic evaluation are dark in colour , coarse , and characteristic Odour , bitter taste are the parameter

Behavioral character of *Aegle marmelos* linn leaves (Crude powder) with different reagent:

Table: 5 Behavioral characters of *Aegle marmelos* linn leaves (Crude powder) with different reagent:

s.no	Powder crude drug	Visible light	UV -254 nm	UV -365
1	Powder	Green	Dark green	Green
2	Powder +HCL	Dark green	Brown	Greenish black
3	Powder+ Con.H_2SO_4	Green	Black	Blue
4	Powder + con. HNO_3	Light brown	Dark green	Dark violet
5	Powder + CH_3COOH	Yellow	Dark yellow	Yellow
6	Powder +Naoh	greenish brown	Greenish black	Brown
7	Powder + iodine	Green	Blue	Dark brown
8	Powder + $Fecl_3$	Green	Reddish black	Greenish brown
9	Powder + picric acid	Dark yellow	Yellow	Dark yellow
10	Powder+ ammonia	Light green	Dark green	Greenish brown

The power drug was treated with hcl gives dark green colour in visible light and show brown colour in UV 254 and greenish black colour in UV 365. Powder treated with con sulphuric acid gives green colour visible light , and black colour in UV 254nm, blue colour in UV 365 nm .in conc nitric acid gives light brown colour in visible light , and dark green colour in UV 254 nm , dark violet colour in UV 365 nm .acetic acid gives yellow colour in

visible light ,and Dark yellow colour in UV 254 nm ,yellow fluorescence in UV 365 nm. iodine treated powder gives green colour in visible light, blue colour in UV 254 nm and dark brown colour in UV 365nm. Ferric chloride treated powder gives green colour in visible light, reddishblack colour in UV 254nm, greenish brown colour in UV 365nm. Picric acid treated powder gives dark yellow colour in visible light, yellow colour in UV 254 nm, and dark yellow colour in UV 365 nm. Ammonia treated powder gives light green colour in visible colour, dark green colour in UV 254 nm, greenish brown colour in UV 365nm. These parameter are used for qualitative parameter for to identify the crude powder drug.

Fluorescent analysis of the Extracts:

The behavior of various extracts in natural light and under UV light at 254nm and365nm and presented in **Table.6**

Table 6: Fluorescence analysis of extracts of *Aegle marmelos* linn extract:

Extract	Consistency	Colour	Visible light	UV 254 nm	UV 365 nm
ETHANOL	Sticky	Light green	Light green	Yellowish green	Brown colour
AQUEOUS	Sticky	Dark green	Dark green	Green	Blue colour

From the table, it was observed that the ethanolic, aqueous extracts were colour under UV light at 365nm. The ethanol and water extracts were give the colour under UV light at 365nm. These parameters are useful for quality control and purity checking of the plant in extract form.

PHYTOCHEMICAL EVALUATION:

PRELIMINARY PHYTOCHEMICAL SCREENING

The results obtained for the preliminary phytochemical screening of the aqueous and ethanolic extract of *Aegle marmelos linn* leaves were presented in **Table .7**

Table: 7 Preliminary phytochemical screening of *Aegle marmelos* linn leaf Powder and different extracts:

s.no	Test	Aqueous. Extract	Ethanolic Extract
1	**Alkaloids:**		
	Mayer's test	Negative	Negative
	Dragentroffs	Positive	Positive
	Hager's test	Positive	Positive
	Wagner's test	Positive	Positive
2	**Carbohydrate**		
	Benedicts test	Positive	Positive
	Fehling's test	Positive	Positive
	Moloch's test	Positive	Positive
	Bar fords test	Positive	Positive
3	**Anthraquinone glycoside**		
	Borntragers test	Positive	Positive
	Modified borntragers test	Positive	Positive
4	**Cardiac glycoside**		
	Keller killani test	Negative	Negative
	Raymond test	Negative	Negative
	Legal test	Negative	Negative
5	**Cyanogenic glycoside**	Positive	Positive
6	**Coumarin glycoside**	Positive	Positive
7	**Sterols**		
	Salwoskis test	Negative	Positive

	LibermanBurchard test	Negative	Positive
8	**Sappoins**	Positive	Positive
9	**Tannins & phenolic cpds**		
	$Fecl_3$ test(aqueous)	Positive	Positive
	$Fecl_3$ test (alcoholic)	Positive	Positive
	Gelatin test	Positive	Positive
	$Kmno_4$ test	Positive	Positive
	Gold beaters skin test	Positive	Positive
10	**Flavonoids**		
	Lead acetate	Positive	Positive
	Shinoda test	Positive	Positive
	Acid test	Positive	Positive
	Alkali test	Positive	Positive
11	**Proteins and amino acids**		
	Million's test	Positive	Positive
	Biurets test	Positive	Positive
	Ninhydrin test	Positive	Positive
12	**Terpenoids**	Positive	Positive
13	**Volatile oil**	Positive	Positive
14	**Fixed oil**	Negative	Negative
15	**Gum**	Positive	Positive
16	**Mucilage**	Positive	Positive
17	**Betacyanins**	Negative	Negative
18	**Anthocyanin's**	Positive	Positive
19	**Emodins**	Positive	Positive
20	**Quinone**	Positive	Positive
21	**Resin**	Negative	Positive
21	**Phlobatannins**	Positive	Positive
23	**Coumarin**	Positive	Positive

The preliminary phytochemical screening procedure of the aqueous and ethanolic extract of *Aegle marmelos linn leaves* showed the presence of carbohydrates, alkaloids, protein and amino acids, flavonoids, terpenoids, phenolic compounds, tannins, saponins ,coumarins ,anthocyanins, volatile oil and cardiac glycoside, phytosterols, pholobatannins, and absence of volatile oil ,resin. Sterols.

QUANTITATIVE ESTIMATION OF PHYTOCONSTITUENT:

ESTIMATION OF TOTAL PHENOLIC CONTENT:

The results for the total phenol estimation of ethanolic extract of *Aegle marmelos* linn leaves are tabulated in **Table.8** and **Fig.1**

Fig. 1 Calibration curve of Gallic acid:

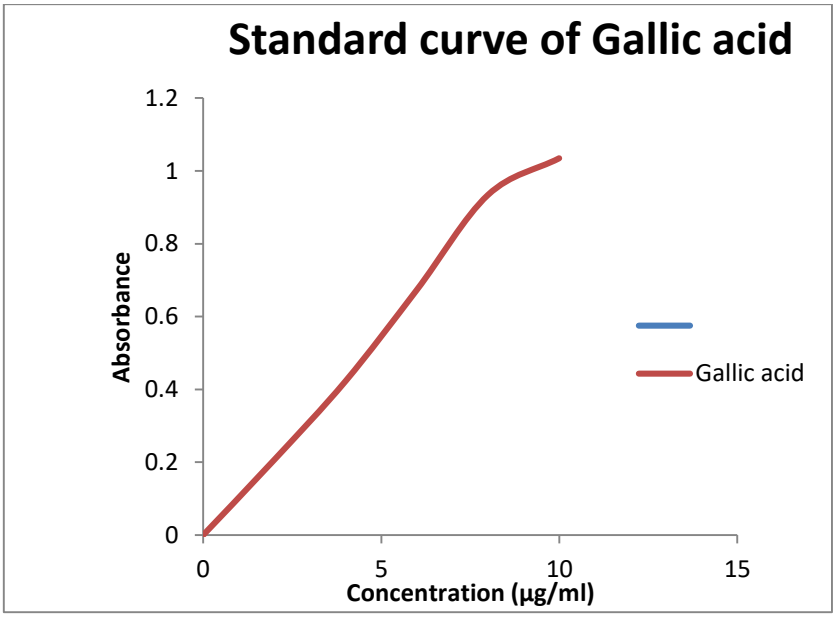


Table .8:Total phenolic content in ethanolic extract *Aegle marmelos* linn leaves of in terms of Gallic acid equivalents

S.no	Conc of Gallic acid mcg/ml	Absorbance at 760 nm	Conc.of extract mcg / ml	Absorbance at 760 nm	Amount of total phenolic content in terms mgGAE/g of extract
1	2	0.2050	10	0.0663	0.5801
2	4	0.4230	20	0.1150	1.0386
3	6	0.6733	30	0.1953	1.773
4	8	0.9343			
5	10	1.035		GAE	56.6mg/g

The linear regression equation was found to be y=0.1087x0.0021 while the correlation coefficient was found to be 0.9998. The amount of phenol content present in the extract in terms mg GAE/g of extract was found to be **56.6 ± 0.338** by using the above linear regression equation.

ESTIMATION OF TOTAL FLAVONOID CONTENT:

The results for the total flavonoid estimation *of* ethanolic extract of *Aegle marmelos* linn leaves are tabulated in **Table .9, fig .2**

Fig.2 : Calibration curve of Rutin

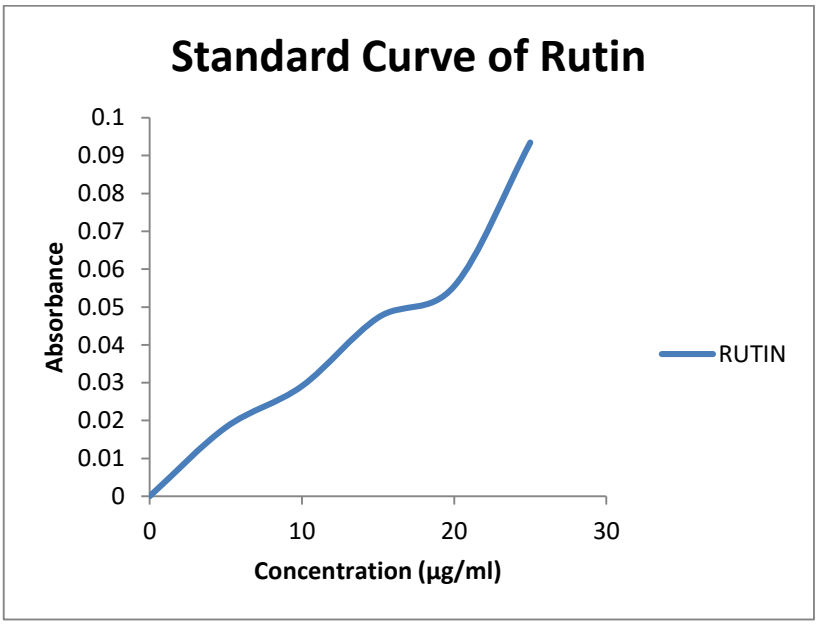


Table .9: Total flavonoid content per gram of extract in terms of Rutin by aluminum chloride method

s.no	Conc of rutin mcg/ml	Absorbance at 415 nm	Conc .of extract mcg / ml	Absorbance at 415 nm	Amount of total flavonoid content in terms mg rutin/g of extract
1	5	0.0182	10	0.00184	1.114
2	10	0.0291	20	0.00364	1.67
3	15	0.0472	30	0.00541	2.3
4	20	0.0555			
5	25	0.0935		RUE	83.5mg/g

The linear regression equation was found to be y=0.0307x-0.0432 while the correlation was found to be 0.9974. The amount of flavonoid content present in the extract in terms mg quercetin equivalent/g of extract was found to be **83.5 ± 0.30** by using the above calibration curve equation.

ESTIMATION OF TOTAL TANNIN CONTENT:

The results for the total tannin estimation of ethanolic extract of *Aegle marmelos linn are* tabulated in **Table 10 , Fig.3**

Fig. 3 : Calibration curve of Tannic acid

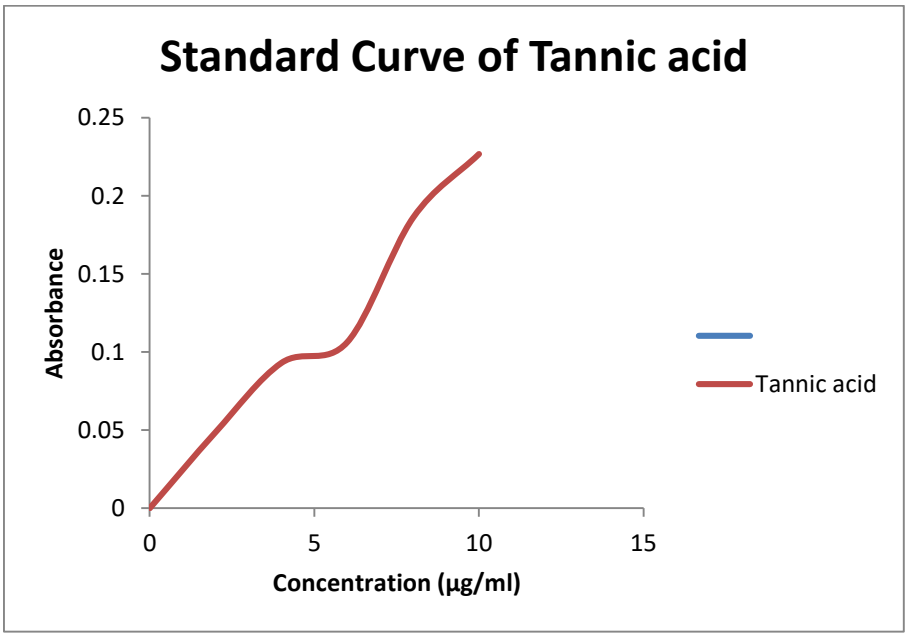


Table .10: Total tannin content in ethanolic extract of *Aegle marmelos* linn in terms of Tannic acid equivalents

s.no	Conc of tannic acid mcg/ml	Absorbance at 760 nm	Conc .of extract mcg / ml	Absorbance at 760 nm	Amount of total tannin content in terms mg tannic acid/g of extract
1	2	0.0487	10	0.0093	0.4708
2	4	0.093	20	0.0193	0.9237
3	6	0.1063	30	0.03417	1.5829
4	8	0.186			
5	10	0.2267		Tannin eq	49.62 mg/g

Total tannin determination is carried out by spectrophotometry after oxidation of the analyte with the Folin–Denis reagent in alkaline medium. This method is based on a redox reaction and other reducing agents in the samples. The linear regression equation was found to be y = 0.0223x - 0.0012 while the correlation was found to be 0.9997. The amount of tannin content present in ethanolic extract of aegle marmelos linn in terms of mg tannic acid/g of extract was found to be **49.62 ± 0.12** by using the above calibration curve equation.

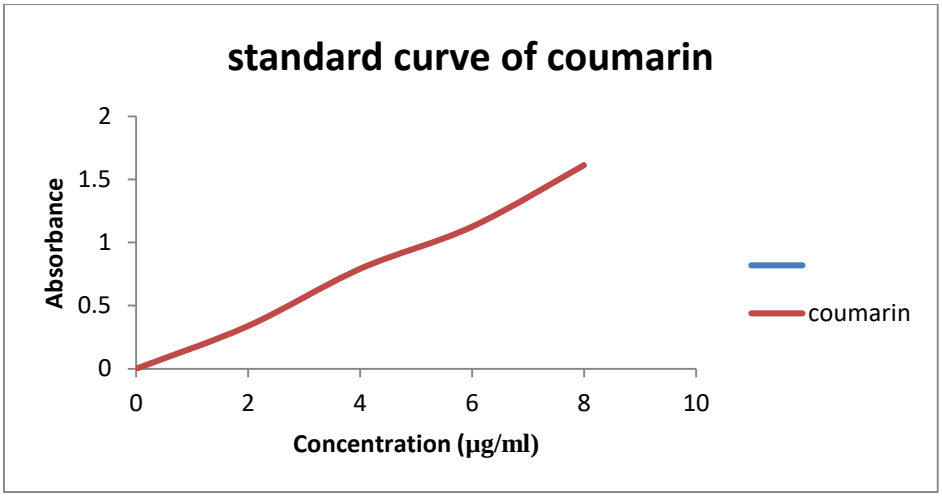
ESTIMATION OF TOTAL COUMARIN CONTENT:

The results for the total coumarin estimation of ethanolic extract of *Aegle marmelos linn* are tabulated in **Table 11 , Fig .4**

Table.11: Total coumarin content in ethanolic extract of *Aegle marmelos linn* in terms of coumarin equivalents

s.no	Conc of coumarin mcg/ml	Absorbance at 320 nm	Conc of extract mcg / ml	Absorbance at 320 nm	Amount of total tannin content in terms mg tannic acid/g of extract
1	2	0.3393	4	0.342	1.9908
2	4	0.7913	6	0.469	2.6023
3	6	1.1246	8	0.757	3.9889
4	8	1.6123			
5	10	0.2267		coumarin eq	47.67 mg/g

Fig .4 : calibration curve of standard coumarin



The total coumarin content determination is carried out by spectrophotometry by ionization phenolic hydroxyls in the molecules by alkaline hydroxide causes a bathochromic deviation. The linear regression equation was found to be y = 0.2005x - 0.0286 while the correlation was found to be 0.9997. The amount of coumarin content present in ethanolic extract of *Aegle marmelos* linn in terms of mg coumarin /g of extract was found to be **47.67 ± 0.12** by using the above calibration curve equation

Pharmacological activity of medicinal plants is ascribed to the quality and quantity of biologically active compounds they contain. Quantitative phytochemical screening of leaf powder and extracts of *Aegle marmelos* linn revealed the presence of biologically active compounds like, flavonoid ,coumarin , tannin, phenolic compounds , are accredited to antibacterial, anti-oxidant, anti-inflammatory, antitumor, skin diseases and in treatment of diabetic. .

Quantitative estimation of biological compounds showed that EEAM linn leaves extract has more amount flavonoid content (83.5 mg/g) , followed by phenolic content (56.6±o.338) , coumarin content (47.67±0.12) , and tannin content was (49.2±0.12). This could be used as diagnosis the nature and amount of phytoconstituents.

CHROMATOGRAPHY

THIN LAYER CHROMATOGRAPHY:

The number of spots, Rf value of the EEAM and the colour of the spots under UV light 365nm is presented in **Table .12** and the photograph of the plate is

Presented in **plate .10&11.**

Table.12: TLC Profile of *Aegle marmelos* linn leaves:

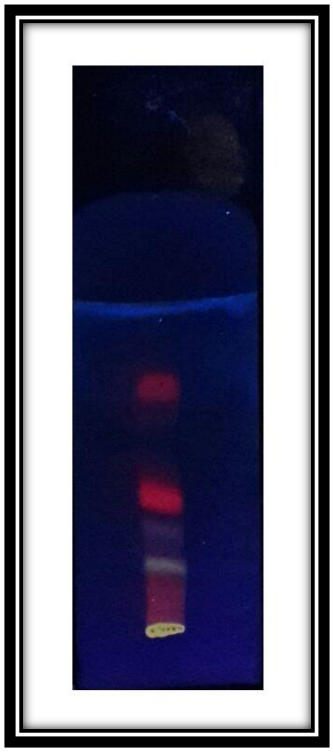
S.no	Mobile phase	Number of spots	Colour of spots at 365 nm	Rf value
1.	Toluene :ethyl acetate: formic acid (8:2:0.01)	3	Light pink	0.38
			Red	0.46
			Violet	0.62
2.	Benzene: acetone (9:1)	2	Brilliant violet	0.47
			Light pink	0.39

The extract showed 3 spots at 365nm. The Rf value of 0.62, 0.38 and 0.46may be due to the presence of flavonoids, phenolic compounds and coumarin. When viewed under UV at 365m after development in the mobile phase namely Benzene: Acetone (9:1). The Rf value of 0.39 and 0.4 may be due to the presence of coumarin glycoside.

PLATE NO: 10 TLC Profile of EEAM

MOBILE PHASE: BENZENE: ACETONE (9:1)

PLATE NO:11 TLC Profile of EEAM



MOBILE PHASE: TOLUENE: ETHYL ACETATE: FORMIC ACID (8:2:0.01)

ISOLATION AND CHARACTERISATION OF BIOLOGICALLY ACTIVE COMPONENT FROM ETHANOLIC LEAF EXTRACT OF *AEGLE MARMELOS* LINN:

Column chromatography of Ethanolic leaves extract of *Aegle marmelos* linn (Table –13)

A mixture of chloroform- ethanol fraction of ethanolic leaf extract of *Aegle marmelos* leaf extract was evaporated under vacuum and purified by using methanol.. After purification a yellow crystalline powder was obtained. And it was identified and validated by various analytical parameters like solubility, melting point, chemical test and spectroscopic methods.

TABLE-13

Data showing the column chromatography of EEAM

EEAM			
Fractions	**Elutes**	**Nature of residue**	**No .of spots**
1-5	Pet.ether (30ml)	Pale green	-
6-7	Pet.ether :Chloroform 20:10 (ml)	Dark Green	-
8-9	chloroform (20ml)	Green	-
9-12	Chloroform :ethanol 9:1 (20m)l	Yellowish green	2
13-14	Chloroform:ethanol 4:1 (20ml)	Brownish green	3
15-17	Chloroform:ethanol 2:1(20ml)		2

Nature and properties of isolated compound from chloroform –ethanol fraction of EEAM

Table.14

S.no	Properties	Compound 1
1	Nature	Crystalize
2	Colour	Yellow
3	Melting point	72^0C
4	Refractive index v	0 .
5	Solubility	Very soluble in ether, di-ethyl ether, chloroform and ethanol.

IDENTIFICATION AND THINLAYER CHROMATOGRAPHIC STUDIES OF ISOLATED COMPOUND:

Thin layer chromatography:

The Rf value of isolated compound and colour of the spot under UV -365 nm reported in **table.15** and photograph of plate presented in **plate.12**

S.no	Mobile phase	Number of spots	Colour of at 365 nm	Rf value
1.	Benzene: acetone (9:1)	1	Orange	0.37

PLATE. NO: 12

TLC IMAGES OF ISOLATED COMPOUND VISULALIZED AT 365nm

Benzene: Acetone (9:1)

Chemical test:

The chloroform :ethanol fraction were concentrated and the chemical test were performed ,the result are tabulated in table.16 .

Table .16:chemical test for isolated fraction chloroform:ethanol fraction

S.no	Test	Observation	Inference
1.	Fraction +10% amm.hydroxide	Intense fluorescence	Due to the presence coumarin
2.	Few ml of fraction +alcoholic $fecl_3$+conc HNO_3	First green colour formed then turned to yellow colour	Due to the presence coumarin
3.	Few ml of fraction + 1 N Naoh	Development of green fluorescence	Due to the presence coumarin

The above result indicates the presence of coumarin compound and its derivatives.

SPECTRAL STUDIES ON ISOLATED COMPOUND:

UV SPECTROSCOPY:

The UV spectral analysis of compounds isolated from the hexane fractions of were recorded using UV spectroscopy was presented in (**Table.17),** Fig.5

Table.17 UV-Spectrum of Isolated Compound

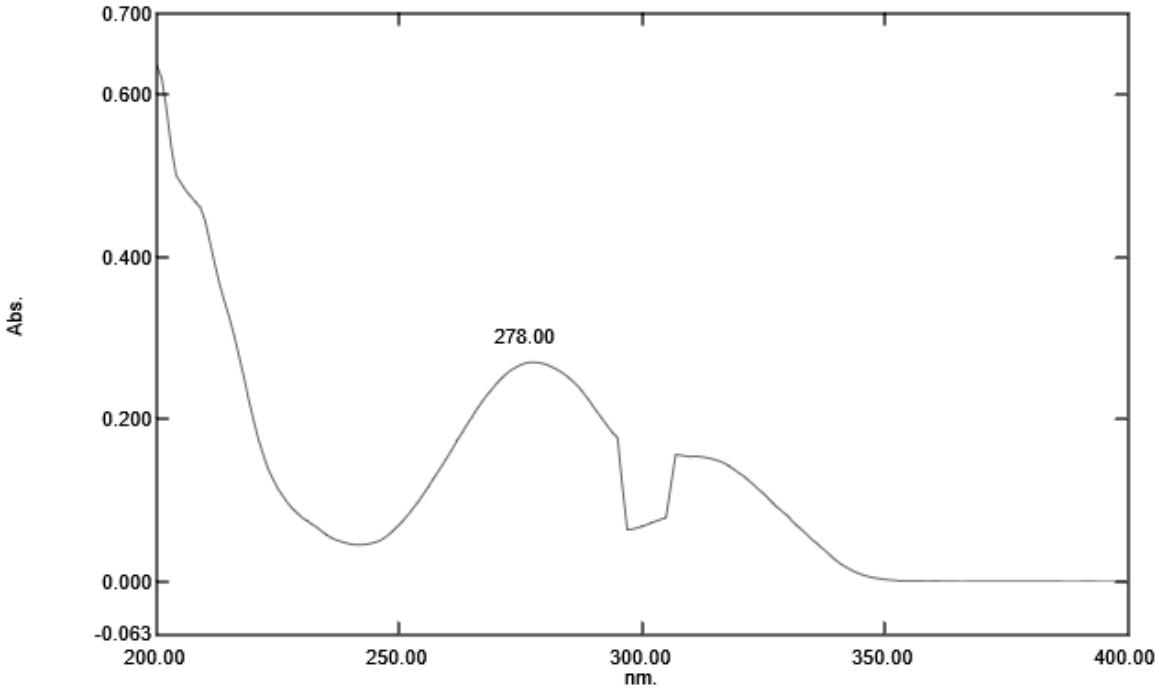


Table .17 absorption spectrum value of isolated compound from EEAM.

s.no	Wavelength	Absorbance
1.	278	0.268
2.	310	0.154

The UV spectrum of compound isolated from the fraction of chloroform: ethanol from the leaf ethanolic extract of *Aegle marmelos* linn leaves. Showed the maximum absorbance of 0.268nm and 0,154nm at ƛmax of 278 and 320nm respectively. When compared with UV spectrum of standard coumarin (0,194 at **ƛmax of 278,325).(Harbone J B1998)** From this findings were exposed the UV spectrum of isolated compound from EEAM chloroform: ethanol fractions were gave similar to that of standard. Hence, we concluded that the isolated compounds can be coumarin.

Infrared spectroscopy

Infrared spectroscopy of isolated compound from the chloroform:ethanol fractions of EEAM. The results were given in **Table.11 and Fig .6**

Fig.IR Spectrum of isolated compound from chloroform : ethanol fraction of EEAM

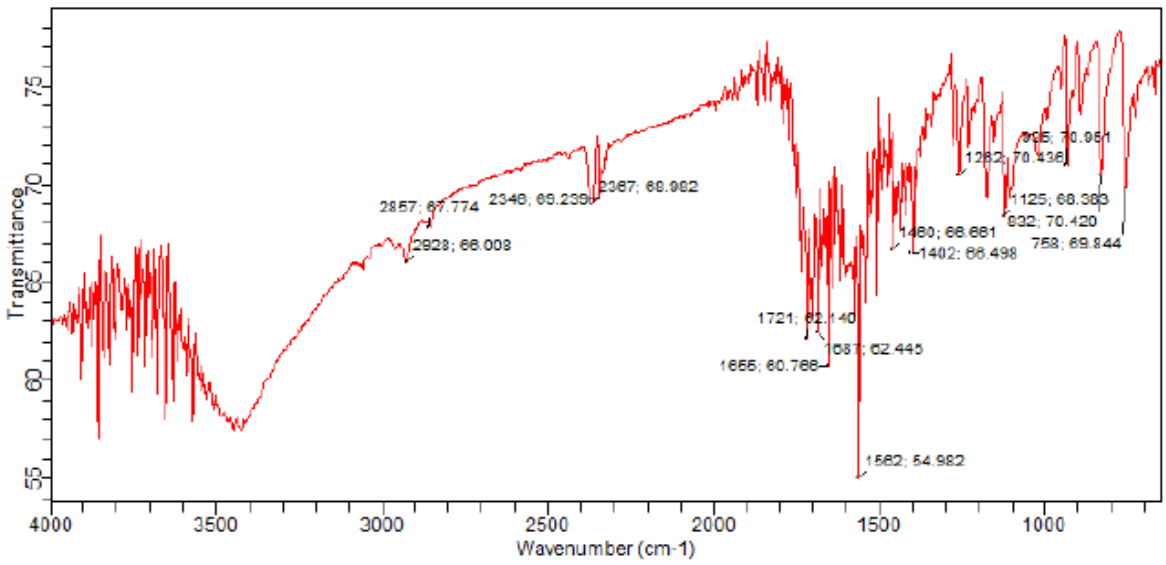


Table.18: The IR spectrum observed ranges of isolated compound from EEAM

S.no	Observed IR range cm^{-1}	Functional group	Types of band
1.	3381 cm^{-1}	OH	Stretching
2.	2963 cm^{-1}	Aromatic CH	Stretching
3.	1715 cm^{-1}	Co carbonyl group	Stretching
4	900-600 cm^{-1}	Aromatic CH	Bending
5.	1400-1600 cm^{-1}	C=C	Stretching

The IR spectrum of chloroform: ethanol fraction isolated compound shows lactone carbonyl at 1715 cm-1, ν C=C at 1608 cm-1, electromagnetic radiation in the infrared region of the spectrum which results in changes in the vibrational energy of molecules.. it was compared with standard and the purity of the compound is similar to standard coumarin.

The IR spectrum of (chloroform –ethanol fractions of EEAM) compound along with standard coumarin shows the following similarities

Alcohol O-H stretching

Carboxyl group

C=C stretching

C- H stretching From this findings were exposed the IR spectrum of isolated compound from EEAM chloroform: ethanol fractions were gave similar to that of standard. Hence, we concluded that the isolated compounds can be coumarin.(Harbone J B 1998)

3. PHARMACOLOGICAL ACTIVITY:

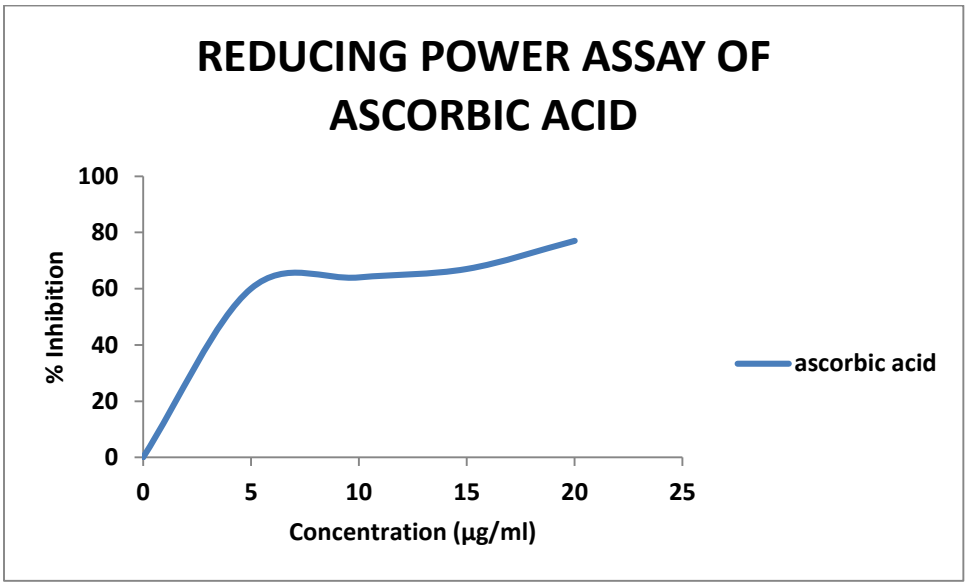
Antioxidants and Health

Antioxidants are man-made or natural substances that may prevent or delay some types of cell damage. Diets high in vegetables and fruits, which are good sources of antioxidants, have been found to be healthy; however, research has not shown antioxidant supplements to be beneficial in preventing diseases. Examples of antioxidants include vitamins C and E, selenium, and carotenoids, such as beta-carotene, lycopene, lutein, and zeaxanthin.

Rigorous scientific studies involving more than 100,000 people combined have tested whether antioxidant supplements can help prevent chronic diseases, such as cardiovascular diseases, cancer, and cataracts. In most instances, antioxidants did not reduce the risks of developing these diseases

METHOD 1: REDUCING POWER ASSAY

The result obtained from the reducing power assay of ethanolic extract of *Aegle marmelos* linn leaves and standard ascorbic acid are presented in **table.19** and the graphical representation is presented in **fig. 6**



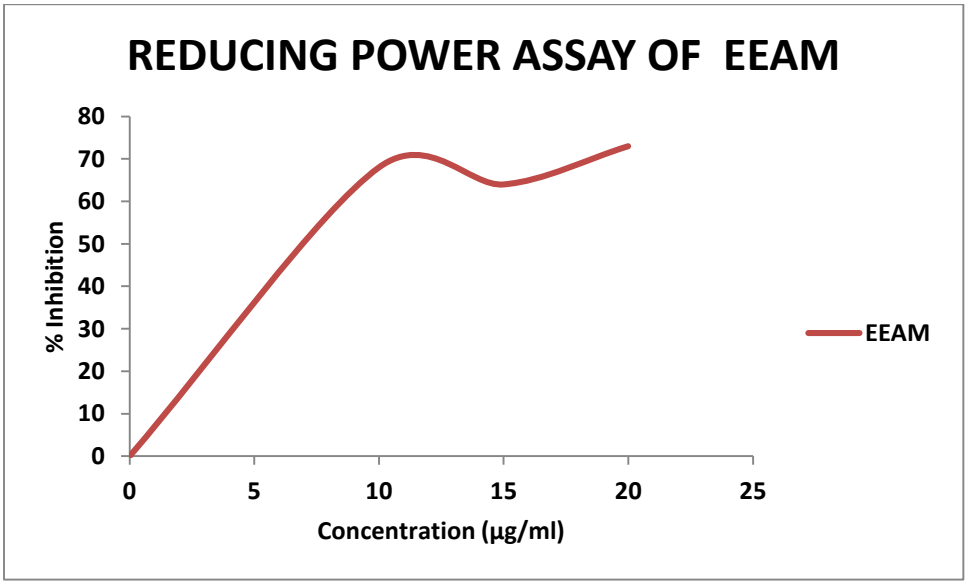


Table:19 **Absorbance of ethanolic extract of *Aegle marmelos* linn leaves and standard ascorbic acid in reducing power assay:**

S .no	Concentration		Percentage inhibition of ascorbic acid	Percentage inhibition of *Aegle marmelos linn* mcg / ml
	Ascorbic acid mcg/ml	Aegle marmelos linn mcg/ml		
1	5	10	60± 0.33	64± 0.57
2	10	15	64± 0.88	68± 0.66
3	15	20	67± 0.57	73±0.33
	20		77±0.33	
		IC_{50} value	9 mcg /ml	12 mcg/ml

The inhibitory concentration (IC_{50}) ethanolic extract *Aegle marmelos* linn leaf against reducing power assay determined in comparison with ascorbic acid used as a standard. The inhibitory concentration (IC_{50}) of *Aegle marmelos* linn leaves in reducing power assay was found to be 12 mcg/ml in comparison with ascorbic acid 9 mcg/ml. the concentration ethanolic extract of *Aegle marmelos* linn showed the moderate antioxidant effect.

METHOD 2: TOTAL ANTIOXIDANT ACTIVITY BY PHOPHOMOLYBDNUM METHOD

The result obtained from the phosphomolybdenum method assay of ethanolic extract of *Aegle marmelos* linn leaves and standard ascorbic acid in are presented in **table** .20: and the graphical representation is presented in **fig.15**

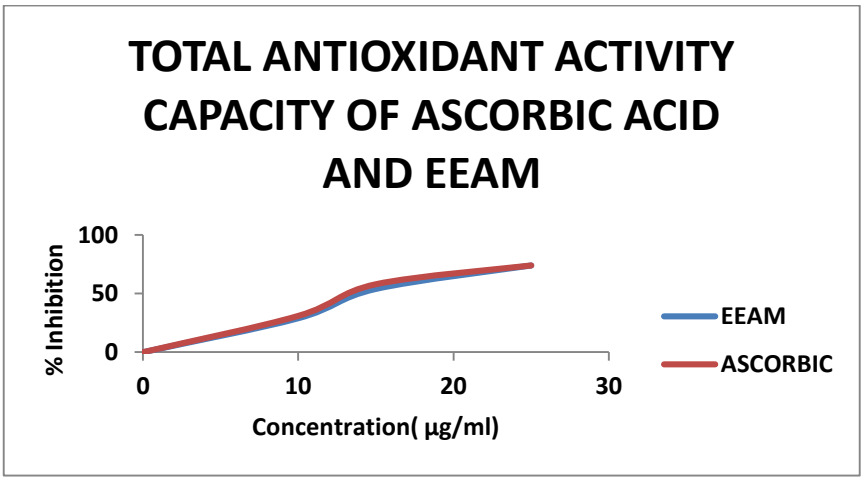


Table:20: **Absorbance of ethanolic extract of *Aegle marmelos* linn leaves and standard ascorbic acid in phosphomolybdenum assay**

S .no	Concentration		Percentage inhibition of ascorbic acid mcg /ml	Percentage inhibition of *aegle marmelos linn* mcg / ml
	Ascorbic acid mcg/ml	Aegle marmelos linn mcg/ml		
1	10	10	31± 0.27	29± 0.47
2	15	15	58± 098	54± 0.72
3	25	25	79± 0.54	74± 0.88
		IC_{50} value	14 mcg /ml	16 mcg /ml

The inhibitory concentration (IC_{50}) ethanolic extract *Aegle marmelos* linn leaf against Total antioxidant capacity determined in comparison with ascorbic acid used as a standard. The inhibitory concentration (IC_{50}) of *Aegle marmelos* linn leaves in Total antioxidant capacity was found to be 16 mcg/ml in comparison with ascorbic acid 14 mcg/ml. the concentration ethanolic extract of *Aegle marmelos* linn showed the moderate antioxidant effect.

INVITRO: SUNPROTECTION FACTOR DETERMINATION BY ULTRA VIOLET SPECTROSCOPY:

SPF Number indicates the time period for the product up to which it protects the person while stay in the sun before burning.in order to protect the skin against ultraviolet radiation.in the present work ethanolic extract of *Aegle marmelos* linn leaves were subjected for spf evaluation by ultraviolet spectroscopic method .the calculated spf value ethanolic extract of *Aegle marmelos* linn leaves are tabulated.

The result obtained from the sun protection factor method of ethanolic extract of *Aegle marmelos linn* leaves are presented in **Table .21 to .24**

Table: 21 SPF of ethanolic extract of *Aegle marmelos linn* leaves:

For 2 mcg /ml concentration:

s.no	Wavelength (nm)	EE*I(Normalized)	Absorbance value of EEAM	SPF Value
1	290	0.0150	0.005±0.002	0.00075
2	295	0.0187	0.0463±0.026	0.0378
3	300	0.2874	0.0767±0.044	0.2204
4	305	0.3278	0.1047±0.060	0.3432
5	310	0.1864	0.1283±0.074	0.2391
6	315	0.0839	0.1463±0.084	0.1227
7	320	0.0180	0.1543±0.089	0.0277
			SPF	0.99±0.05

Table. 22: SPF of ethanolic extract of *Aegle marmelos linn* leaves:

FOR 4 mcg/ml concentration:

s.no	Wavelength (nm)	EE*I(Normalized)	Absorbance value of EEAM	SPF Value
1	290	0.0150	0.0127±0.007	0.0019
2	295	0.0187	0.0527±0.03	0.0430
3	300	0.2874	0.0837±0.048	0.2405
4	305	0.3278	0.1137±0.065	0.3727
5	310	0.1864	01350±0.077	0.2516
6	315	0.0839	0.1530±0.088	0.1283
7	320	0.0180	0.1577±0.091	0.0283
			SPF	1.066±0.40

Table: 23: SPF of ethanolic extract of *Aegle marmelos linn* leaves:

For 6 mcg/ml concentration:

s.no	Wavelength (nm)	EE*I(Normalized)	Absorbance value of EEAM	SPF Value
1	290	0.0150	0.0156±0.009	0.0023
2	295	0.0187	0.1573±0.09	0.1285
3	300	0.2874	0.2357±0.13	0.6774
4	305	0.3278	0.3137±0.18	0.0315
5	310	0.1864	0.4133±0.23	0.7703
6	315	0.0839	0.5133±0.29	0.4306
7	320	0.0180	0.475±0.27	0.0855
			Spf	2.125±0.80

Table. 24: SPF of ethanolic extract of *Aegle marmelos linn* leaves:

Standard 25 mcg/ml:

S.no	Wavelength (nm)	EE*I(Normalized)	Absorbance value of EEAM	SPF Value
1	290	0.0150	0.412±0.53	0.0618
2	295	0.0187	0.467±0.17	0.0873
3	300	0.2874	0.659±0.43	1.893
4	305	0.3278	0.870±0.27	2.851
5	310	0.1864	0.984±0.26	1.8374
6	315	0.0839	1.234±0.33	1.035
7	320	0.0180	0.968±0.64	0.1742
			Spf	7.939±0.99

SPF Number indicates the time period for the product up to which it protects the person while stay in the sun before burning.in order to protect the skin against ultraviolet radiation.in the present work ethanolic extract of *Aegle marmelos* linn leaves were subjected for spf evaluation by ultraviolet spectroscopic method .the calculated spf value ethanolic extract of *Aegle marmelos* linn leaves are tabulated,

The spf value of ethanolic extract of *Aegle marmelos* linn leaves 2 mcg/ml are **0.99±0.05**, 4 mcg/ml have spf value about **1.066±0.40**,6 mcg /ml spf value about **2.125±0.80**.the marketed sunscreen product have a concentration 25 mcg / ml shows spf value about **7.93± 0.99.** .it indicates that the ethanolic extract of *Aegle marmelos* linn shows good sun protection activity against UV radiation. It may be linked to the antioxidant and photo protective activities observed in extract. The presence of flavonoid, coumarin compounds will absorb the maximum absorbance range at 240-280 and 290 325 nm. So the extract also proved to be interesting for designing new studies aiming incorporation of the extract into photo protective cosmetic formulation.

IN VIVO UVA PHOTOPROTECTIVE EVALUATION ON EEAM LINN USING ZEBRAFISH EMBRYO ASSSAY:

PRIMARY TOXICLOGICAL STUDY USING ZEBRAFISH EMBRYO:

The maximal acceptable toxicant dose of EEAM linn leaves were studied on the early development zebrafish embryo and the result are tabulated in **table no.25 to 27**

Table .25: SCORE FOR THE WHOLE EMBRYO TOXICITY FOR EEAM:

Conc. mcg/ml	Hours	Score
0.5	12	Nil
	24	Nil
	36	Nil
	48	Nil
0.75	12	Nil
	24	Nil
	36	Nil
	48	Nil
1	12	Nil
	24	1
	36	Nil
	48	1
2	12	Nil
	24	1
	36	Nil
	48	1

Table .26: SCORE FOR THE WHOLE EMBRYO FOR DMSO:

Conc.	Hours	Score
O.1%	12	Nil
	24	Nil
	36	Nil
	48	Nil

Table .27: SCORE FOR THE WHOLE EMBRYO FOR STANDARD CAFFEINE:

Conc.	Hours	Score
10 mcg/ml	12	Nil
	24	Nil
	36	Nil
	48	40

Effect of EEAM on the development stages of zebra fish embryo was carried out. The maximal acceptable toxicant concentration (MATC) was calculated by scoring the malformation .0.1% DMSO and standard caffeine (10 mcg/ml) were used as a control and standard toxin. No malformation and mortality observed up to 0.5 to 0.75 mcg/ml concentration level(score).but 1-2 mcg/ml concentration observe the swollen of egg , pigmentation , bent tail up to 48 hpf.no mortality observed in this concentration level .Total mortality observed in the standard caffeine at 10 mcg/ml concentration (40).

IN VIVO UVA PHOTOPROTECTIVE EVALUATION ON EEAM LINN USING ZEBRAFISH EMBRYO ASSSAY:

The effects of UVA radiation induced morphological changes on zebrafish embryos and the protective effect of EEAM linn leaves are shown in **Plate.13- 17** result are tabulated in **Table** .28 to 31

Table.28: Control:

Malformation	12 hrs	24 hrs	36 hrs	48 hrs
Swollen of egg	--	-+	++	++
Abnormal pigmentation	--	++	++	++
Tail malformation	--	--	--	++

Table .29:Positive control:

Malformation	12 hrs	24 hrs	36 hrs	48 hrs
Swollen of egg	--	++	++	++
Abnormal pigmentation	--	++	++	++
Tail malformation		--	--	++

Table .30: Standard:

Malformation	12 hrs	24hrs	36hrs	48hrs
Swollen of egg	--	--	--	--
Abnormal pigmentation	--	--	--	-+
Tail malformation	--	--	--	+-

Table .31:EEAM 0.75 mcg / ml:

Malformation	**12 hrs**	**24 hrs**	**36 hrs**	**48 hrs**
Swollen of egg	--	--	--	--
Abnormal pigmentation	--	--	-+	-+
Tail malformation	--	--	--	++

(--) indicates the no malformation

(++) indicates the malformation occur (max)

(-+) indicates the malformation occur (min)

The prospective photo protective properties of ethanolic extract of *Aegle marmelos* linn was determined by initially assessed the toxicity of these extracts at the concentration used for UV photo protection and found the no viability and malformatio. On zebra fish embryo. The *Aegle marmelos* linn ethanolic extract showed the efficacy at a concentration of 0.75 mcg/ml, shows 75% effectiveness to protect zebrafish embryos against UVA photo

INVIVO UVA PHOTOPROTECTIVE EVALUATION ON EEAM LINN LEAVES:

Standard 10 mcg/ml:

Time : 24 hrs 36 hrs 48 hrs

PLATE.NO:13

EEAM 0.75 mcg/ml:

24 hrs 36 hrs 48 hrs

PLATE.NO:14

DMSO 0.1%:

PLATE.NO:15

Control:

PLATE.NO:16

Normal devolpment:

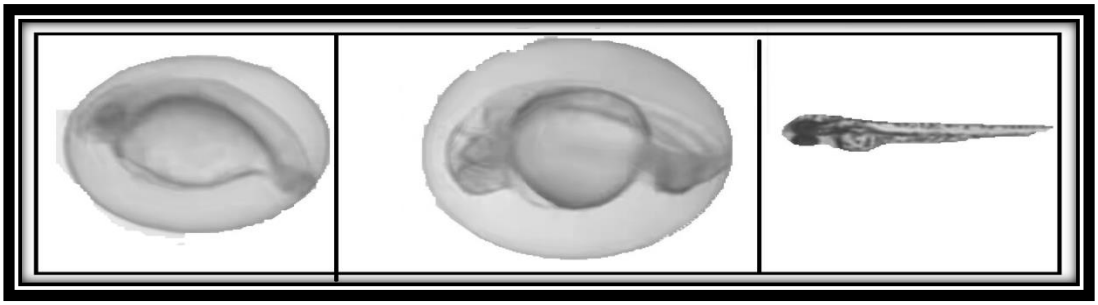
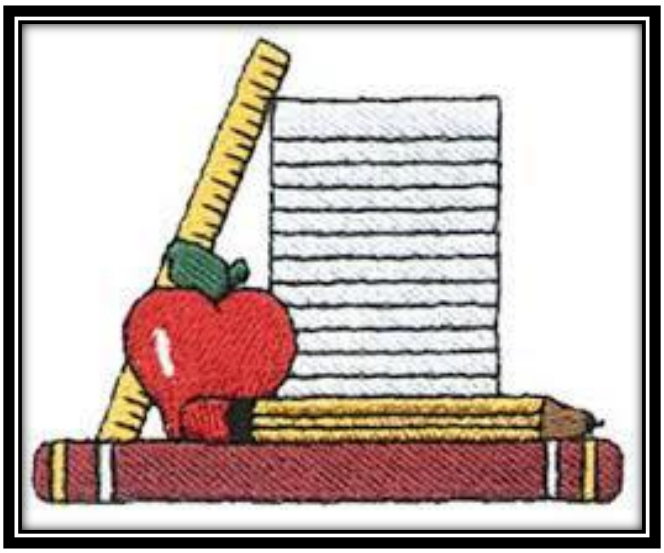


PLATE.NO:17

damage. Under these conditions none of the extracts considerably affected the viability of the embryos, therefore, we concluded that EEAM involved in the study, at a concentration of 0.75 mcg/ml, were safe and non-toxic for their use with zebrafish embryos. The effects of UVA and extracts on zebrafish embryos are shown in plate.

The EEAM protect the UVA radiation induced morphological changes in zebrafish embryo due to their moderate antioxidant activity compared to the other parts of the plant due to the presence of high amount of phenolic, flavonoid, coumarin content. These compound enhance the photo protection (inhibit the free radical formation, and neutralize the reactive oxygen species) from the harmful UV radiation. From the previous data and corresponding study were elucidated the isolated compound. Its widely used for various biological activity such as anti-coagulant, anti-microbial, vasodialator, sedative, and photosensitivity. It contributes the photo protective effect ethanolic extract of *Aegle marmelos* attain by the presence of coumarin and its derivatves.(Raphael et al., 2015)

CHAPTER-6



SUMMERY AND CONCLUSION

SUMMARY AND CONCLUSION

The present study entitled the **IDIOSYNCRATIC APPORCH FOR AN ERRATIC AND STUNNING MANAGEMENT OF *Aegle marmelos* linn LEAVES ON PHOTOSENSITIVITY BY USING ZEBRAFISH EMBRYO MODEL** focuses on a plant which is commonly available throughout India and traditionally used in treatment of various ailments.

Studies on the leaves of *Aegle marmelos* linn are still lacking. Hence to exploit its potential use encouraged the present study to investigate the leaves of this plant with clear scientific protocol.

The chapter on **Literature Review deals** with the information regarding the pharmacognostical, phytochemical and pharmacological evaluation of the *Aegle marmelos* plant and other species of *Aegle*.

The chapter on **Pharmacognostical studies** highlights on

Macroscopical features were studied and the adherence of general characters to the *Aegle marmelos* linn was found. The leaves *Aegle marmelos* linn are trifoliate; pellucid-punctate surface and showing parallel venation with brittle texture; lateral subsessile and it terminal long petiole. These morphological tools are supportive for identification, authentication, and distiquish from the relevant species of the genus.

The microscopial examination lamina portion of the T.S of *A. marmelos* leaf showed the presence of upper and lower epidermis both comprised of round to oval shaped cells. Both upper and lower epidermal layers bear stomata. Each stoma has two guard cells and two subsidiary cells and they correspond to rubiaceous type. Numbers of stomata are high in upper epidermis compared to lower epidermis and both epidermis shown presence of covering trichomes. Interior to the epidermis is a many layered palisade tissue, which consists of closely, packed oval cell without much intercellular space. Midrib portion shows the presence of xylem and phloem arranged in an arc.

Quantitative microscopical studies namely stomatal number, stomatal index, vein islet number, vein termination number and physiochemical evaluation such as ash value, extractive value, loss on drying value etc.,

Also studied cell powder microscopy, fluorescence analysis, organoleptic evaluation of powder and the results helps in achieving a trouble-free identification and authenticity of the plant leaf or in powder form in future.

The chapter on **Phytochemical Evaluation** deals with

Preliminary phytochemical screening reveals the presence of carbohydrate, alkaloids, coumarin flavanoids, protein & aminoacids, glycosides etc.,

Quantitative determination of secondary metabolites (phenol, flavanoid, tannin, coumarin

content) has been carried out.

TLC studies showed the presence of flavanoid, phenolic, coumarin compound.

The isolation of coumarin from chloroform: ethanol fraction of EEAM leaves extract by using column chromatography. And it's were identified by using chemical test, thin layer chromatography, spectral studies with standard.

The chapter on **Pharmacological studies** focuses

The Antioxidant activity by various methods and the extract possessed a good antioxidant property due to the presence of poly phenolic, flavonoid content.

The In vitro sun protection factor of ethanolic extract of *Aegle marmelos* linn leaves was determined by UV visible spectrometer. The spf value of ethanolic extract of *Aegle marmelos* linn leaves 2 mcg/ml are **0.99±0.05**, 4 mcg/ml have spf value about **1.066±0.40**,6 mcg /ml spf value about **2.125±0.80**.the marketed sunscreen product have a concentration 25 mcg / ml shows spf value about **7.93± 0.99**. The extract shows good sun protection activity against UV radiation. It may be linked to the photo protective activities observed in extract. So the extract also proved to be interesting for designing new studies aiming incorporation of the extract into photo protective cosmetic formulation. The activity due to the presence flavonoid, phenolic, compound will maximum absorption at the UV radiation ranges.

The In vivo Photo protective activity against UVA radiation of EEAM was evaluated using zebrafish embryo assay. The toxicity of ethanolic extract of *Aegle marmelos* linn used for photo protective activity was initially assessed by whole embryo toxicity study. The concentration 0.75 mcg/ml was safe and non-toxic for their use with zebrafish embryos. The *Aegle marmelos* linn ethanolic extract showed the efficacy at a concentration of 0.75 mcg/ml, shows 75% effectiveness to protect zebrafish embryos against UVA photo damage. from the previous data and corresponding study were elucidated the isolated compound. It's widely used for various biological activity such as anti-coagulant, anti-microbial, vasodialator,sedative , and photosensitivity. It contributes the photo protective effect ethanolic extract of aegle marmelos attain by the presence of coumarin and its derivatives.

From the above findings, it concluded that the further investigation may be carried out for the bio active fraction for its potential pharmacological effects. New innovation on the development formulation for inflictive on photosensitiasation of uv radiation.

future research studies may be extended to isolate other phyto constituents present in this plant. Besides the above findings, it would be more appropriate to augment further research on clinical trials for improving the plant based drug industry and the development of new drugs of herbal origin for photosensitivity, globally emerged diseases and disorders.

CHAPTER-7

REFERENCE

- Adhami VM, Syed DN, Khan N, Afaq F. Phytochemicals for prevention of solar ultraviolet radiation induced damages. Photochem Photobiol, 2008; 84:489-500.
- Afaq F, Mukhtar H. Photo chemoprevention by botanical antioxidants. Skin Pharmacol Appl Skin Physiol, 2002; 15:297–306.
- Ali, Muhammad shaiq , Pervez , Muhammad kashif , marmenol : a 7- geranyloxy coumarin from the leaves of aegle marmelos corr. Natural product research, vol 18,(2) , 2004, 141-146.
- Amirtham,S., Jeyanthi,P and Settu,A Phytochemical and Anti-cholestrol activity of *Aegel marmelos (L.)* Corr. leaf extract using in albino mice Int.J.Curr.Microbiol.App.Sci (2016) Special Issue-3: 65-74
- Amit Pandey and Rashmi Mishra ,2011 Antibacterial properties of *Aegle marmelos* leaves, fruits and peels against various pathogens Jpbms, 2011, 13 (13)
- Andley UP, Becker B, Hebert JS, Reddan JK, Morrison AR, Pentland AP. Enhanced prostaglandin synthesis after ultraviolet-B exposure modulates DNA synthesis of lens epithelial cells and lowers Intraocular pressure *in vivo*. Invest Ophthalmol Vis Sci, 1996; 37:142-153
- Applied Thin-Layer Chromatography: Best Practice and Avoidance of Mistakes, 2nd Edition. Edited by Elke Hahn-Deinstrop. Copyright © 2007 WILEY-VCH VerlagGmbH &Co. KGaA,Weinheim.
- Ariharan V.N.and Nagendra Prasad P :Mahavilva - a sacred tree with immense medicinal secrets: a mini review, Rasayan J Chemistry. 2013 ;Vol. 6 (4): 342 - 352

- Arul, V., Miyazaki, S. and Dhananjayan, R. Mechanisms of the contractile effect of the alcoholic extract of *Aegle marmelos* Corr. on isolated guinea pig ileum and tracheal chain. Phytomedicine, 2004; 11: 679-683.
- Arunachalam,D., Kantha,D., Subhashini,S.,Sathesh kumar,A., Wound healing and antigenotoxic activities of *Aegle marmelos* with relation to its antioxidant properties .Journal of pharma res ;2012,5(3),1492-1502.
- Attimarad MKK, Ahmed M and Harsha S. High Performance Thin Layer Chromatography. A powerful analytical technique in pharmaceutical drug discovery. Pharm methods. 2011; 2(2): 71-75.
- Badam, L., Bedekar, S.S., Sonawane, K.B., Kiran, B. and Joshi, S.P. In vitro antiviral activity of bael (*Aegle marmelos* Corr) upon human coxsackieviruses B1- B6. Journal of Communicable Diseases, 2002; 34(2): 88-99.
- Balakumar.S, Rajan.S, Thirunalasundari.T, Jeeva .S , Asian pacific journal of tropical biomedicine , 2011, 1, 309-312.
- Barthakur, N.N., Arnold, N.P. Cetrain organic and inorganic constituents in bael (*Aegle marmelos*) fruit. Tropical Agriculture (Trinidad), 1989; 66: 65-68.
- Basu, D. and Sen, R. Alkaloids and coumarins from root-bark of *Aegle marmelos*. Phytochemistry, 1974; 13: 2329-2330.
- Behavioral and neurotoxic effects of arsenic exposure in zebrafish (Danio rerio, Teleostei:
- Behera, J., Mohanty, B., Ramani, Y., Rath, B. and Pradhan, S. Effect of aqueous extract of *Aegle marmelos* unripe fruit on inflammatory bowel disease. Indian Journal of Pharmacology, 2012; 44(5): 614-618.

- Bele A.A. and Khale A. (2011). Standardization of herbal drugs: A review. International Journal of Pharmacy.2(12): 56-60
- C.K. Kokate, K.R. Khandelwal, A.P. Pawer& S. B. Gokhale, Practical Pharmacognosy, 3rd edition, NiraliPrakashan, Pune, 1995, 137.
- Chakthong, S., Weaaryee, P., Puangphet, P., Mahabusarakam, W., Plodpai, P., Voravuthikunchai, S.P. and Kanjana-Opas, A. Alkaloid and coumarins from the green fruits of *Aegle marmelos.* Phytochemistry, 2012; 75: 108-113.
- Chapter from the book phytochemicals –A Global perspective of their role in nutrtion and health.www.intechopen.com
- Chatterjee, A. and Mitra, S.S. On the constitution of the active principles isolated from the matured bark of *Aegle marmelos* correa. Journal of the American Chemical Society, 1949; 71(2): 606-609.
- Chatwal GR and Anand SK .Instrumental Methods of Chemical Analysis. 2009; 2: 566-68.
- Chauhan A , Agarwal M, Kushhwaha S, mutreja. A,. Supression of fertility in male albino rats following the administration of 50% ethanolic extract of aegle marmelos .Contraception 2007;76,474-481.
- Cosa, ., Vlietinck A.., Berghe, DV., Maes, L. (2006). Anti-infective potential of natural products: How to develop a stronger in vitro 'proof-of-concept'.Ethnopharmacol. 106: 290–302..
- De Castro, M. R.; Lima, J. V.; de Freitas, D. P.; Valente Rde, S.; Dummer, N. S.; de Aguiar, Cyprinidae). Comp. Biochem. Physiol. C. Toxicol. Pharmacol. **2009,** 150, 337-342.

- Dinesh Kumar Sekar, Gaurav Kumar, L. Karthik and K. V. Bhaskara Rao. A review on pharmacological and phytochemical properties of *Aegle marmelos* (L.) Corr. Serr. (Rutaceae)Asian Journal of Plant Science and Research, 2011; 1(2): 8-17.
- Dineshkumar C. Pharmacognosy can help minimizeaccidental misuse of herbal medicine. CurrSci 2007;3:1356-1358.
- Eberhardt, T.L., Li, X., Shupe, T.F. and Hse, C.Y. (2007). Chinese Tallow Tree (SapiumSebiferum) utilization: Characterization of extractives and cell-wall chemistry. Wood Fiber Sci. 39: 319-324.
- Fabricant, D.S. and Farnsworth, N.R. (2001). The value of plants used in traditional medicine for drug discovery. Environ. Health Perspect. 109, 69–75.
- Farooq S.: Medicinal plants- Field and laboratory manual. Dehradun, International Book Distributers, 2005; 40-42.
- Gaur R D, *Flora* of the district Garhwal North West Himalaya (with ethnobotanical notes), (Trans Media, Srinagar Garhwal), 1999; 811.
- Gautam, M.K., Ghatule, R.R., Singh, A., Purohit, V., Gangwar, M., Kumar, M., Goel, R.K. Healing effects of *Aegle marmelos* (L.) correa fruit extract on experimental colitis. Indian Journal of Experimental Biology, 2013; 51(2): 157-164.
- Gavimath c.c , Y.L Ramachandra, S.P Rai, H.V.Sudeep, P.S.S Ganapathy, B.T Kavitha, asian journal of bio science , 2008,3,333-336.
- George K V, Mohanan N & Nair SS, Ethnobotanical investigations of *Aegle marmelos* (Linn.) Corr. in: Ethnobotany and Medicinal Plants of India and Nepal, by Singh V and Jain AP, (Scientific Publishers, Jodhpur), 2003; 29-35.

- Gopalsamy Rajiv Gandhi, Savarimuthu Ignacimuthu, Michael Gabriel Paulraj, Hypoglycemic and β-cells regenerative effects of Aegle marmelos (L.) Corr. bark extract in streptozotocin-induced diabetic rats. Food Chem Toxicol 2012 May 30;50(5):1667-74. Epub 2012 Jan 30.
- Gruijl FR. Photo carcinogenesis: UVA vs UVB. Methods Enzymol, 2000; 319:359-366.
- Harbone J B, Phytochemical Methods- A Guide to Modern Techniques of Plant Analysis, Chapman and Hall Ltd,London, 1998, 42, 129, 189, 203.
- Harbone J B, Phytochemical Methods, Chapman and Hall Ltd, London, 1973, p. 49-188.
- Ichihashi M, Ando H, Yoshida M, Niki Y, Matsui M. Photoaging of the skin. Anti-Aging Medicine, 2009; 6:46-59.
- Jagessar RC, Cox M (2010) Phytochemical screening of the CHCl3 and CH3CH2OH extract of stems, twigs, roots, and barks of *Conocarpus erectus* L. Int J Acad Res 2: 36-45.
- Jagetia, G.C., Venkatesh, P. and Baliga, M.S. Evaluation of the radioprotective effect of bael (*Aegle marmelos*) extract in mice. International Journal of Radiation Biology, 2006; 80: 281-290.
- Jain UK and Dixit VK. Spectrophotometric estimation of tannins from the Chyavanprash. Indian drugs. 2004; 41: 469-472.
- Jayanthi P and Lalitha P, Reducing Power of the Solvent of Eichhornia crassipes*(Mart.)* Solms, Indian J of Pharmacy and Pharm Sci. 2011; 3(3): 0975-1491.

- Joshi P. Fish stupefying plants employed by tribals of southern Rajasthan- a Probe. Curr. Sci, 1986; 55: 647-650.
- Jyoti M Benni , Jayanthi MK,Suresha RN.Evaluation of the anti-inflammatory activity of *Aegle marmelos* (Bilwa) root. Indian J Pharmacol 2011 Jul; 43(4):393-7
- Kakiuchi, N., Senaratne, L.R.E., Huang, S.L., Yang, X., Hattori, M., Pilapitiya, U. and Namba, T. Effects of Constituents of Beli (*Aegle marmelos*) on Spontaneous Beating and Calcium-Paradox of Myocardial Cells. Planta Medica, 1991; 57: 43-46.
- Kamalakannan N, Prince PS , Hypoglycemic effect of water extract of *Aegle marmelos* fruits in streptozocin diabetic rats. Journal of enthopharmacology,2003;87, 207-210.
- Katiyar S. Silymarin and skin cancer prevention: anti-inflammatory, antioxidant and immunomodulatory effects (Review). Int J Oncol, 2005; 26:169-76.
- Kaur P, Walia A, Kumar S, Kaur S. Antigenotoxic activity of polyphenolic rich extracts from Aegle marmelos (L.) Correa in human blood lymphocytes and E. coli PQ 37. Rec Nat Prod 2009; 3(1): 68-75.
- Keuker H *et al*. in "Proceedings of the International Symposium on Instrumental Thin Layer Chromatography", Brighton, Sussex, UK. 1989; 105-114.
- Kokate C.K.,Purohit A.P. andGokhale S.B. (1994). Pharmacognosy. NiraliPrakashan, Pune13th Edition: 593-597.
- Kokate CK, Practical Pharmacognosy, NiraliPrakashan,Pune, India, 4th Edn, 2000;107-111.
- Kokoski J, Kokoski R, Salma FJ. Fluorescence of powdered vegetable drugs under ultraviolet radiation. J Am Pharm Ass 1958; 47:715-717.

- Kothari, S., Mishra, V., Bharat, S. and Tonpay, S.D. Antimicrobial activity and phytochemical screening of serial extracts from leaves of *Aegle marmelos* (Linn.). *Acta Poloniae.* Pharmaceutica-Drug Research, 2011; 68(5): 687-692.
- Kritikar K R and Basu B D, Indian Medicinal Plants, Vol I-IV (Bishen Singh Mahendra Pal Singh, Dehradun), 1984; 830.
- Krutmann J. The role of UV rays in skin aging. Eur J Dermatol, 2001; 11:170-171.
- Laloo D and Sahu AN. Antioxidant activities of three Indian commercially available Nagakesar: An *in-vitro* study, J Chem Pharm Res. 2011; 3(1): 277-283.
- Mabry TJ, Markham KR, Thomas MB. The systematic identification of flavanoids. Springer Verlay New York USA. 1970.
- Maheswari, VL, Joshi, PV and Patil , RH (2009); In vitro anti diarrhoel activity and toxicity profile of aegle marmelos correa ex.roxb. dried fruit pulp , natural product radiance; 8(5), 498-502.
- Maity, P., Hansda, D., Bandyopadhyay, U. and Mishra, D.K. Biological activities of crude extracts and chemical constituents of bael, *Aegle marmelos* (L.) Corr. Indian Journal of Experimental Biology, 2009; 47(11): 849-861.
- Manandhar, M.D., Shoeb, A., Kapil, R.S. and Popli, S.P. New alkaloids from *Aegle marmelos*. Phytochemistry, 1978; 17: 1814-1815.
- Manoj A Suva* Evaluation of sun protection factor of zingiber officinale Roscoe Extract by ultraviolet spectroscopy method, journal of pharmsci tech 2014;3(2).
- Maria Guinea , Virginia Franco , Lidia Araujo-Bazan , Ivan Rodriguez-Martin , Salvador Gonzalez. In vivo UVB-photoprotective activity of extracts from commercial marine Macroalgae, Food and Chemical Toxicology 50 (2012) 1109–1117

- Mazumder, R., Bhattacharya, S., Mazumder, A., Pattnaik, A.K., Tiwary, P.M. and Chaudhary, S. Antidiarrhoeal evaluation of *Aegle marmelos* (Correa) Linn. root extract. Phytotherapy Research, 2006; 20(1): 82-84.
- Mishra AK , Mishra A , Chattopadhyay P Herbal cosmeceuticals for photoprotection from ultraviolet B radiation : A review .Trop j pharm res , June 2011;10(3):354.
- Mohammad A. Kaisar, Mohammad S. Rahman, Mohammed Z. Rahman,Choudhury M. Hasan1 and Mohammad A. Rashid A .AReview on Phytochemicals from Some Medicinal Plants ofBangladesh.Journal of Pharmacy and Nutrition Sciences, 2011, 1, 87-95.
- Nadeem, A.S., Mohd, M., Abdul, K.N and Mohd, A (2010). Evaluation of antioxidant activity, quantitative estimation of phenols and flavonoids in different parts of Aegle marmelos. *African Journal of Plant Science*., 4(1): 001–005.
- Nagani K, Kaneria M, Chanda S. Pharmacognosticstudies on the leaves of ManilkarazapotaL.(Sapotaceae). Phcog J 2012; 4(27):38-41.
- Nandkarni, A. K. Indian Materia Medica. Vol.-I, (3rd ed.). Popular Prakashan, Bombay, 1976; 45-49.
- Nisha RRS, Radhamany PM. Pharmacognostical andphysicochemical analysis on the leaves of Brunfelsia Americana L. AsianPac J Trop Biomed 2012; S305-307.
- Ohashi K, Watanabe H, Ohi K, Arimoto K and Okumura Y (1995). Chemistry let. 881.
- Osorio o k ., Martins J L S.Determinacao de coumarina em extrato fluid e tintura de guaco porespectrometeria derivida de primeria ordem.Brazilian journal pharmaceutical sci,2004;40 (4) 481-486.

- Parmar C & Kaushal M K, Wild Fruits of the sub-Himalayan Region, (Kalyani Publishers, New Delhi), 1982; 136.
- Phuwapraisirisan P, Puksasook T, Jong-aramruang J, Kokpol U. "Phenylethyl cinnamides: A new series of alfa-glucosidase inhibitors from the leaves of Aegle marmelos." Bioorganic & Medicinal Chemistry Letters 2008; 18, 4956-4958.
- R. B, dos Santos, L. C.; Marins, L. F.; Geracitano, L. A.; Monserrat, J. M.; Barros, D. M. Rahman A Said HM, Ahmad VU (1986). Pakistan encyclopaedia planta medica. Hamdard foundation press.
- Rahul Kasar1*, AshishGogia, Kartik Shah2, Vetriselvan1 and ChinmayAnandCritical Factor That Governs a Successful TLC/ HPTLC Analysis of Herbal Medicinal Products (HMPS).RRJPA | Volume 2 | Issue 4 | October-December, 2013, 1-8.
- Rajbir Bhatti , Singh,J,MP,Ishar,S. Pharmacognostic standardisation and antiproliferative activity of *Aegle marmelos* (L.) Correa leaves in various human cancer cell lines. Indian J Pharm Sci 2013 Nov;75(6):628-34
- Rana, B.K., Singh, U.P. and Taneja. V. Antifungal activity and kinetics of inhibition by essential oil isolated from leaves of *Aegle marmelos*. Journal of Ethnopharmacology, 1997; 57: 29-34.
- Raphael,F, luz,Ivo JC, Vieira, RaimundoBraz-Filho,Vinicius ,moreria. C^{13} NMR data from coumarins from moraceae family, american journal of analytical che,2015,6.851-866.
- Rattan k , Basak , prabir K , Mandal, Amal K , Mukherjee , "studies on neutral polysaccharide isolated from the aegle marmelos fruit pulp''carbohydrate research , vol 97(2), 1981,315-321.

- Rerio); University of Oregon Press: 1995.
- Ritu Gupta, International Journal of Herbal Medicine, 2016; 4(4): 114-115.
- Robak J, Gryglewski RJ. Bioactivity of flavonoids.Pol J Pharmacol 1996; 48:555-564.
- Sabu.MC and kuttan, R Anti diabetic activity of aegle marmelos and its relationship with its antioxidant properties. Indian journal of physiology pharmacology, 2004:48(1):81-88.
- Sahare KN, Anadharaman V , Meshram SU, Reddy MV , Tumane PM , Anti-microfilarial activity of methanolic extract of vitex negundo and *Aegle marmelos* and their phytochemical analysis . Indian journal of experimental biology.2008;46(9022):128-131.
- Saxena AP, Vyas KM. Ethnobotanical records on infection diseases from tribals of Banda district. J Econ Tax Bot. 1981; 2:191-194.
- Sevugan ,A., Subramanian .,k Balamuthu., K .,Abdul, B.A.A., Mohamed, A.A and Mandali., V.R(2008) Anti diabetic activity of leaf and callus extract of aegle marmelos in rabbit. science Asia., 34:317-318.
- Shankaranath V.,Balakrishanan,N, Suresh.D.,Sureshpandian .G ,Edwin. E, Sheeja . E, Fitoterapia 2007, 78, 258-259.
- Sharma, B.R., Rattan, R.K. and Sharma, P.Constituents of *Aegle marmelos*. Indian Journal of Chemistry, 1980; 19: 162.
- Shoeb, A., Randhir, S.K., Popli, S.. Coumarins and alkaloids of *Aegle marmelos.* Phytochemistry, 1973; 12: 2071- 2072.

- Shyam S Agrawal, Ashok Kumar, Sumeet Gullaiya, Vishal Dubey, Ashish Nagar, Poonam Tiwari, Parul Dhar, Antifertility activity of methanolic bark extract of Aegle marmelos (l.) in
- Siddiqui & M.A. Hakim, Format for the pharmacopoeial analytical standards of compound formulation, workshop on standardization of Unani drugs, January, Central Council for Research in Unani Medicine (CCRUM), New Delhi, (appendix), 1995, 24-25.
- Sivaraj s, A Balakrishnan, M.Thenmozhi ,R.Venkatech , journal pf pharmacy research 2011,4,1507-1508.
- Srivastava (ed.) MM, High performance thin layer chromatography (HPTLC), Springer-VerilogBerlin Heidelberg 2011.
- Sugeng, R., Mohd, A.S., Mawardi, R., Gwendoline, C.L.E., Taufiq, Y.Y.H., Norio, A and Mariko, K (2001).Alkaloids from Aegle marmelos (Rutaceae). Malaysian Journal of Analytical Sciences., 7(2) : 463–465.
- Sukhdev AR: A Selection of Prime Ayurvedic Plant drugs Ancient- modern concordance Anamaya Publicatons, Br J Surg, 1975; 62: 542-552.
- Svobodová A, Psotová J, Walterová D. Natural phenolics in the prevention of UV-induced skin damage. Biomed Papers, 2003; 147:137– 145.
- Umashankar, Govindappa M, Yarappa Lakshmikantha R, Padmalatha Rai S and Channabasava. Isolation and Characterization of Coumarin Isolated from Endophyte, Alternaria Species -1 of Crotalaria pallida and Its Apoptotic Action on HeLa Cancer Cell Line, Metabolomics,2015;5(4).

- United States Pharmacopeia and National Formulary, USP 25, NF 19, (2002). United States Pharmacopeial Convention Inc., Rockville.
- V.Krishnan Nambiar, P.Jayanthi, and T.K Samu, E-Journal of Indian Medicine, **13,**73(2000)
- Vanitha P Reddy Antioxidant properties and stability of aegle marmelos leaves extracts. J Food Sci Technol 2013 Feb 26;50(1):135-40. Epub 2011 Jan 26
- Veerappan A K, Srinivasan & Renganathan D Corr. on amphibian heart in-situ preparation, Proc 6th Internet World Congress for Biomedical., *Cardiotonic effect of Aegle marmelos* Sciences, 2000.
- Vignesh Sundararajan,Induja kalyana sundram,Deepika Deeptirekha sarangi. Poly herbal formulation with anti-elastase and anti-oxidant properties for skin anti-aging. Altern Med 2018 Jan 29;18(1):33.
- Vikas Kumar, Danish Ahmed, Amita Verma, Firoz Anwar Umbelliferone β-D-galactopyranoside from Aegle marmelos (L.) corr. An ethno medicinal plant with ant diabetic, antihyperlipidemic and ant oxidative activity. BMC Complement Altern Med 2013 Oct 20;13:273. Epub 2013 Oct 20.
- Westerfield, M. The Zebrafish Book: A Guide for the Laboratory use of Zebrafish (Danio
- Yadav, NP 2009 phytochemical and pharmacological profile of leaves aegle marmelos linn Review,2009;144-150.

THANK YOU